**University of Dundee**

**MASTER OF SCIENCE**

# Pilot Study Examining the Effects of Remote Ischaemic Preconditioning on Arterial Stiffness and Endothelial Function in Patients with Peripheral Arterial Disease

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# Pilot Study Examining the Effects of Remote Ischaemic Preconditioning on Arterial Stiffness and Endothelial Function in Patients with Peripheral Arterial Disease


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# 4 DECLARATION

“I declare to be the author of this thesis, the content of which is my own work, unless otherwise stated, and has not previously been submitted for any other assessment or higher degree. The report is written in my own words and conforms to the University of Dundee’s Policy on plagiarism and academic dishonesty. Unless otherwise indicated, I have consulted all the references cited in this report”

# 5 ABSTRACT

Background: Intermittent Claudication (IC) is pain on walking that is related to poor blood supply to the lower limbs. Endothelial dysfunction is thought to play a role in the onset and progression of this condition. The idea that short periods of non-lethal ischaemia followed by periods of reperfusion, in one organ or tissue, triggers endogenous protective pathways in other organs is known as Remote Ischaemic Preconditioning (RIPC). RIPC has been shown to improve endothelial function by increasing bioavailability of NO. The study aims to evaluate whether RIPC can improve markers of vascular function by assessing its effect in participants with IC compared to healthy volunteers.

Methods: Seventeen participants were recruited and underwent four days of RIPC protocol. Vascular function was assessed using (i) iontophoresis with Laser Doppler Imaging (LDI), (ii) Post occlusive reactive hyperaemia test (PORH) (iii) pulse wave analysis (PWA) and pulse wave velocity (PWV) to measure arterial stiffness. Blood samples were also taken to assess for any change in serum cytokines and markers of oxidative stress.

Results: Multiple regression analysis demonstrated a statistically significant improvement in PWA, after RIPC intervention, in healthy volunteers only ($p \leq 0.05$ is used as statistical significance). There were no other significant predictors of change in vascular function after RIPC.

Conclusion: RIPC appears to show improvement in arterial stiffness as measured by PWA. The results suggest that RIPC has a greater beneficial effect in healthy volunteers as opposed to patients with IC. The results do not support the idea that improvements in vascular function are solely mediated through improvement in endothelial function.

# 6 LIST OF ABBREVIATIONS

| | |
|---|---|
| ACh | Acetylcholine |
| AIx | Augmentation Index |
| CABG | Coronary Artery Bypass Grafting |
| cGMP | Cyclic Guanosine Monophosphate |
| CK-MB | Creatine Kinase-Myocardial Band |
| CRP | C-Reactive Protein |
| CVD | Cardiovascular Disease |
| DVT | Deep Vein Thrombosis |
| EDHF | Endothelium-derived Hyperpolarising Factor |
| eNOS | Endothelial Nitric Oxide Synthase |
| FMD | Flow-Mediated Dilatation |
| FLPI | Full Field Laser Perfusion Imager |
| GTP | Guanosine triphosphate |
| HDL | High Density Lipoproteins |
| HV | Healthy Volunteer |
| IC | Intermittent Claudication |
| IF | Incidental Findings |
| IL-1 | Interleukin-1 |
| IL-6 | Interleukin-6 |
| IL-8 | Interleukin-8 |
| IL-10 | Interleukin-10 |
| IPC | Ischaemic Preconditioning |

| | |
|---|---|
| ISF | Investigator Site File |
| KATP | ATP-sensitive potassium channels |
| LDF | Laser Doppler Flowmetry |
| LDI | Laser Doppler Imaging |
| LDL | Low Density Lipoproteins |
| LPLV | Last Patient Last Visit |
| MAPKs | Mitogen-activated Protein Kinases |
| mmHg | Millimetres of Mercury |
| nNOS | Neuronal Nitric Oxide Synthase |
| NO | Nitric Oxide |
| NOS | Nitric Oxide Synthase |
| NOX | Nicotinamide-Adenine Dinucleotide Phosphate Oxidase |
| PAD | Peripheral Arterial Disease |
| PIS | Participant Information Sheet |
| PORH | Post-Occlusive Reactive Hyperaemia |
| PU | Perfusion Units |
| PVD | Peripheral Vascular Disease |
| PKC | Protein Kinase C |
| PWA | Pulse Wave Analysis |
| PWV | Pulse Wave Velocity |
| QC | Quality Control |
| RCT | Randomised Controlled Trial |
| R&D | Research and Development |
| REC | Research Ethics Committee |

| | |
|---|---|
| RIPC | Remote Ischaemic Preconditioning |
| RISK | Reperfusion Injury Salvage Kinase |
| ROS | Reactive Oxygen Species |
| SAE | Serious Adverse Event |
| SAFE | Survivor Activating Factor Enhancement |
| SFA | Superficial Femoral Artery |
| SMF | Study Master File |
| SMG | Study Management Group |
| SNP | Sodium Nitroprusside |
| SOP | Standard Operating Procedures |
| STAT3 | Signal Transducer and Activator of Transcription 3 |
| TNF-$\alpha$ | Tumour Necrosis Factor Alpha |

# 7 INTRODUCTION

## 7.1 CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) is a major health burden worldwide and within the UK. In 2014 cancer was the leading cause of mortality in the UK, accounting for 29% of all deaths. CVD was responsible for 27% of all deaths in the UK which equates to a total of approximately 155,000 deaths in the year(1). Although CVD represents a heavy burden of disease the number of deaths as a result of CVD has been declining steadily each year(1). A large part of this is as a result of lifestyle modification and improved medical management. Peripheral arterial disease (PAD) is a term used to describe a condition whereby blood flow to the extremities is compromised or reduced. Most commonly it is the legs that are affected. Blood flow is disrupted by narrowings, called stenoses, or occlusions within an artery. In the majority of the population it is atherosclerotic disease which is responsible for this. One of the most common clinical manifestations of atherosclerotic disease in the lower extremities is pain on walking, or Intermittent Claudication (IC), as it is generally referred to in a clinical setting. Intermittent claudication is acute onset pain, on exertion, that resolves on resting. Patients can experience pain on walking in the calf, thigh, buttocks or a combination of these sites. The site where pain is experienced is usually related to the site of disease within the arterial network. Arterial occlusions or stenoses tend to be above the site of pain i.e pain in the calf muscles is usually as a result of occlusion / stenosis of the common femoral, superficial femoral or popliteal arteries. Pain in the thigh or buttocks is usually related to disease in the aorta or iliac arteries. The incidence of PAD in the UK is about 3-10% in people aged 70 or less and approximately 15-20% in those over 70 years of age(2). Most patients are asymptomatic, while 1 in 10, present with symptomatic disease in the form of IC. In symptomatic patients 3-5% will end up requiring a major amputation(3). A healthy functioning endothelium plays an important role in vascular tissue homeostasis and endothelial dysfunction is associated with more severe cardiovascular disease and peripheral arterial disease(4).

### 7.1.1 Endothelium

The endothelium is a thin monocellular layer that covers the inner surface of the body's arteries. The endothelium, one of the largest organs in the body, is extremely active. It is a paracrine, exocrine and endocrine organ and releases factors that control vascular relaxation and contraction, thrombogenesis and fibrinolysis, and platelet activation and inhibition. The endothelial cell is similar to other cells in the body. A cortical web surrounds the internal surface of the sarcolemma (Figure 1). This cortical web is affected by changes in intravascular tension and responds to increases in intravascular pressure by increasing its stiffness(5). The integrity of the cortical web is important in regulating the adhesiveness of leukocytes and platelets which is important in maintaining vascular homeostasis.

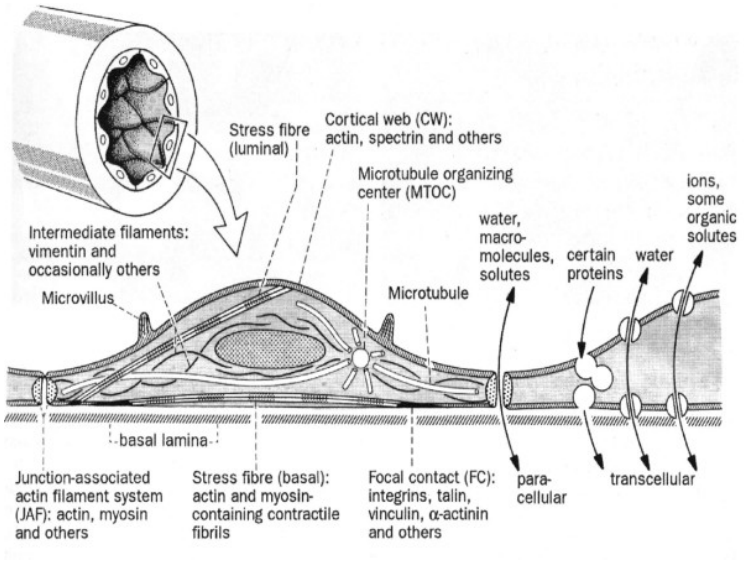



**Figure 1:** General structure of vascular endothelial cell. Adapted from Esper et. al(5)

The major risk factors for CVD, such as hypertension, diabetes, dyslipidaemia and tobacco toxins, are also risk factors for endothelial dysfunction. Diseased endothelial vessel walls become stiffer and their capability for vasodilatation is reduced(6), which hampers the regulatory role that the endothelium provides. Various studies have demonstrated that there is an increased risk of cardiac events in patients with severe coronary endothelial dysfunction despite only mild coronary artery disease and that endothelial dysfunction was also predictive of increased risks of cardiac events

independent of the classic risk factors for coronary artery disease(7)(8). Decreased availability or activity of nitric oxide (NO) can be an early sign of atherosclerosis. Nitric Oxide is a major vasodilator released by the endothelium and is synthesized in endothelial cells from L-arginine by the calcium calmodulin-dependent enzyme nitric oxide synthase (NOS) (9). In a competent blood vessel most nitric oxide is presumed to arise from the activity of endothelial Nitric Oxide Synthase (eNOS) (10) and it is thought that shear stress increases the expression of eNOS. Shear stress is an important haemodynamic factor initiating and driving the inflammatory process. It is felt that low shear stress (<4 dyne/cm2), which is prevalent at sites prone to atherosclerosis, such as arterial bifurcations, can stimulate atherosclerosis(11) whereas at >15dyne/cm2, observed in healthy arteries, an atheroprotective effect is conferred. NO can also pass through the endothelial intima and penetrate the smooth muscular tissue of the arterial wall. NO then, through nitrosilation of the haem from guanylate-cyclase, degrades the guanosine triphosphate (GTP) releasing cGMP. This consequently regulates the cytosolic calcium and causes smooth muscle fibre relaxation and therefore vasodilatation(12).

Nitric oxide performs numerous functions important in maintaining a healthy endothelium. It mediates endothelial dependent vasodilation. It achieves this by opposing angiotensin II and endothelin I. Angiotensin II is formed by hydrolysis of angiotensin 1 via the angiotensin converting enzyme. It initiates its prothrombotic and vasoconstrictive effects via the angiotensin II receptor type 1. Angiotensin II stimulates endothelin converting enzyme which degrades big-endothelin releasing Endothelin-I, which is a powerful vasoconstrictor. NO reduces platelet adherence and aggregation, leukocyte adhesion and infiltration, and, prevents oxidative modification of low-density lipoprotein (LDL) cholesterol(13). Caveolae are flask shaped invaginations in the lumen border of endothelial cells. The plasma membrane of caveolae contain the cholesterol rich Caveolin-1 protein. Caveolin-1 has a specialised scaffolding domain which organises certain interactions between Caveolin-1 and signalling molecules such as eNOS. Caveolin-1 production is increased by oxidised LDL which inactivates eNOS thus inhibiting production of NO(14). It has been suggested the caveolae may compartmentalise signalling in the plasma membrane and that the interactions between resident proteins, such as eNOS, and Caveolin-1 may alter a signalling cascade(15).

Reactive oxygen species (ROS) are thought to be predictive of CVD(16). ROS are produced in the mitochondria by oxidative phosphorylation or by the actions of nicotinamide-adenine dinucleotide phosphate oxidase (NOX) on oxygen free radicals. ROS, such as superoxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$), are produced in many cell types including endothelial cells and vascular smooth muscle cells. The antioxidant enzyme Superoxide dismutase rapidly dismutases $O_2^-$ to $H_2O_2$ which in turn is eliminated by gluthathione peroxidase and catalase to water. Oxidative stress is an imbalance in production of anti-oxidant defences, such as superoxide dismutase, and reactive oxygen species(17). Oxidative stress leads to production of proatherogenic cytokines such as TNF-$\alpha$, adhesion molecules and chemokines, interleukins IL-1 and IL-6 which inhibit eNOS and subsequently production of NO. The pro-inflammatory response drives inflammation which in turn initiates release of more pro-inflammatory cytokines and a vicious cycle is created(5).

## 7.2 PERIPHERAL ARTERIAL DISEASE

As discussed, the symptoms of claudication can be the clinical manifestation of atherosclerotic disease in the lower extremities. When we walk there is an increased oxygen demand to the muscles of the legs. This demand is accommodated for, in part, by vasodilation resulting in augmented blood flow to the muscle groups that require it. Patients with IC have been found to have an impaired ability to react to this increased demand suggesting that there is impaired vasodilation within the vessel. The more severe the disease the more severe the impairment in vasodilation(18). In a trial comparing healthy volunteers with patients with PAD, vasodilation was induced chemically and the resultant vasodilation measured. The superficial femoral artery (SFA) is a common site for atherosclerotic disease which can cause IC. In healthy volunteers the SFA dilated in response to methacholine and nitroprusside but in PAD participants the response was significantly decreased compared to healthy controls(19). There is further evidence that this is related to endothelial dysfunction in a study that measured endothelial dependent flow mediated dilatation (FMD) in participants aged 55 and over and it was found that in the participants with PAD, 60% had an impaired dilatory response compared with 32% in an aged matched control group(20). The same study also found that raised blood glucose, increased ratio of

Low Density Lipoproteins (LDL) to High Density Lipoproteins (HDL) and systolic blood pressure were independent predictors of reduced FMD in PAD subjects. Increased production of ROS and increased levels of oxidative stress have been demonstrated in PAD patients(17)(21) leading to a decrease in the bioavailability of NO. Using antioxidant supplementation to improve endothelial function has shown promise in some studies. Anderson et. al demonstrated improvement in endothelial dependent vasomotor response by administering Lovastatin (lowers LDL) and Probucol (antioxidant)(22). Using L-arginine infusions has been shown to improve walking distance in participants with diagnosed claudication compared to controls and those given an endothelium-independent vasodilator, Prostaglandin $E_1$(23).

## 7.3 REMOTE ISCHAEMIC PRECONDITIONING

The idea that short periods of non-lethal ischaemia followed by periods of reperfusion, in one organ or tissue, triggers endogenous protective pathways in other organs is known as Remote Ischaemic Preconditioning (RIPC). In 1986 Murray et al. (24) demonstrated, in animal models, that inducing brief periods of ischaemia followed by reperfusion in cardiac arteries significantly reduced myocardial infarct size after sustained periods of ischaemia, in what was later termed Ischaemic Preconditioning (IPC). The researchers achieved this by occluding the left circumflex artery, in the hearts of dogs, for 5 minutes and then allowing five minutes of reperfusion. This was repeated 4 times after which the same artery was occluded for a sustained period of 40 minutes causing myocardial infarct. The infarct size in the animals who had been ‘preconditioned’ was found to be 75% smaller than the control group who had not been preconditioned. As interest in this phenomenon grew it was demonstrated by Przyklenk et al. (25) that occlusion/reperfusion of one cardiac artery, the left circumflex, followed by prolonged occlusion of the left anterior descending artery resulted in reduced infarct size. This phenomenon of conferred cardio-protection to a remote site not supplied by the artery submitted to preconditioning was termed Remote Ischaemic Preconditioning (RIPC). Conditioning that occurs before the period of sustained ischaemia is termed Pre-conditioning but research has also focused on initiating preconditioning during the ischaemic event (Per-conditioning) and after the ischaemic event (Post-conditioning).

The effects of RIPC are not just intracardiac. In a swine model blood flow in the hindlegs of pigs was occluded using a tourniquet, in a similar RIPC model, of four cycles of five minutes of occlusion followed by five minutes of reperfusion. After the RIPC was completed the left anterior descending artery in the pigs was subjected to a sustained period of occlusion lasting forty minutes. The infarct size in the pigs subjected to RIPC was significantly less that in the control groups not subjected to RIPC (26). RIPC can be induced by inflating a blood pressure cuff around one of the limbs to a pressure high enough to occlude the targeted artery, pharmacologically (e.g diazoxide) or by peripheral nerve stimulation. Due to its non-invasive nature inflating a blood pressure cuff to a pressure great enough to occlude the target artery is the most straightforward way of inducing RIPC. The exact mechanism of action of RIPC is not fully understood but is thought to be a combination of neural and humoral factors. Where rabbits who have undergone ischaemic preconditioning had their coronary effluent removed and transfused into naïve acceptor hearts. The recipients of the transfusion showed significantly smaller infarct size than control groups (27). Like-wise in animals where a ganglion blocker such as hexamethonium has been administered the cardioprotective effects of RIPC were abrogated in animals who had RIPC induced via transient occlusions of the mesenteric arteries, namely the superior mesenteric artery (28). The RIPC stimulus initiates production of Nitric Oxide and a number of autocoids such as acetylcholine, adenosine, bradykinin, endothelin and opioids, which bind to the appropriate receptors on the plasma membrane of cardiomyocytes, which in turn stimulate a number of signalling pathways that convey a cardioprotective effect to the mitochondria.

As discussed, Nitric Oxide (NO) plays an important role in maintaining a healthy endothelium but NO is also thought to play an important role in RIPC. In the context of RIPC, occlusion of the artery of interest causes shear stress which activates endothelial nitric oxide synthase (eNOS) and in this way levels of NO are increased (29). During ischaemia-reperfusion increased levels of NO have been shown to attenuate oxidative stress and cell apoptosis (30)

Adenosine is thought to play a significant protective signal transduction role in preconditioning. Infusing Adenosine or an Adenosine A1 Receptor agonist was found to reduce infarct size in the hearts of rabbits who were subjected to prolonged

ischaemia similar to that of those who were preconditioned by five minutes of occlusion of a coronary artery followed by ten minutes of recovery (31). Furthermore, they found that an Adenosine receptor antagonist reversed the effects of preconditioning but had no effect on infarct size on a non-preconditioned heart. A1 receptors are Gi-coupled and act to slow the heart rate. Many of these Gi-coupled receptors in the heart can mimic Ischaemic Preconditioning (IPC) and in-fact transient occlusion of coronary arteries has been found to release ligands for adenosine, bradykinin, opioid and sphingosine. It has also been shown that Protein Kinase C (32) and ATP-sensitive potassium channels (KATP) (33) were also involved in the ischaemic preconditioning pathway. Protein Kinase C (PKC) is a key mediator of many pathological and physiological pathways. PKC is made up of 11 phospholipid-dependent serine-threonine kinases classified by their requirement for calcium and diacylglycerol for activation(34). PKC activation is via its translocation to cellular endo-membranes in response to second messengers such as calcium or diacylglycerol, although, some PKC isoforms are activated independent of calcium. PKC has many isoforms which display both beneficial and detrimental effects on endothelial health. Conventional PKC (such as PKC α & ß1/2) translocate on the plasma membrane (Figure 2) and produce a vasoconstrictive effect whereas active PKC-ϵ translocates to the mitochondria and has been shown to have a protective effect on cardiac myocytes(35)

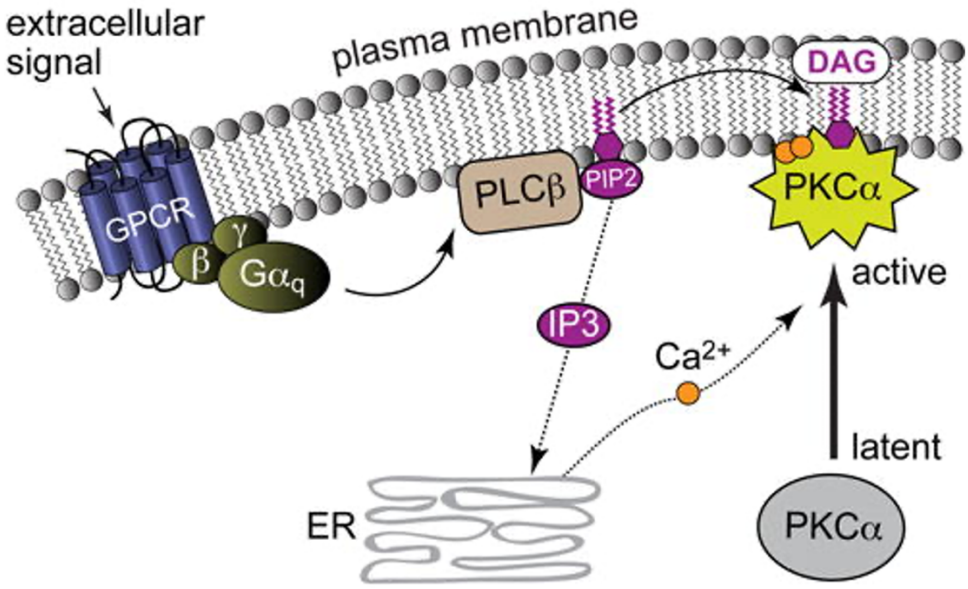



**Figure 2:** Signalling pathway for PKCα at the plasma membrane. Adapted from Igumenova 2015(34)

### 7.3.1 RIPC Pathways

Research has tried to focus on finding a universal signalling pathway to explain the protective effects that Ischaemic Conditioning creates. The Reperfusion Injury Salvage Kinase or Risk Pathway is one proposed by Hausenloy and Yellon (36). Myocardial reperfusion injury is the cell death that occurs in the myocardium after prolonged ischaemia followed by reperfusion. The RISK pathway attempts to describe how a group of survival protein kinases confer powerful cardioprotective effects at the time of myocardial reperfusion (37). The pathway was created to provide a blueprint for the development of targeted therapies to particular sections of the pathway and it has been found that Ischaemic Preconditioning recruits the RISK pathway (38) The RISK pathway has several novel features in that it can be activated by Remote Ischaemic Preconditioning or Postconditioning and that it includes other cardioprotective kinases such as Protein Kinase C, Protein Kinase G, p70s6K and GSK-3$\beta$ (36). It is thought that during the reperfusion stage of RIPC autocoids such as adenosine activate P13K & ERK1/2 which in turn activate eNOS. eNOS catalyses production of NO which stimulates guanylyl cyclase. This inhibits prolonged opening of the mitochondrial permeability transition pores(39).

Another potential pathway that has been shown to be activated by Ischaemic Conditioning is the Survivor Activating Factor Enhancement (SAFE) pathway. One study demonstrated that TNF-$\alpha$ that was given at the start of myocardial reperfusion recruited an alternative signalling cascade termed the SAFE pathway (40). TNF-$\alpha$ has a deleterious effect on the myocardium during reperfusion after an ischaemic event. It is thought that the cardioprotective effects demonstrated are as a result of uptake of TNF-$\alpha$ binding to the TNF receptor 2. This activates the Janus Kinase which recruits signal transducer and activator of transcription 3 (STAT-3) which acts on the mitochondria by modulating mitochondrial respiration and inhibiting mitochondrial permeability transition pores. It has been shown that ischaemic conditioning does activate the SAFE pathway (41). The two pathways have been shown to communicate with each other as they are both thought to be activated by sphingosine-1-phosphate (S1P) (42). Both pathways converge in the mitochondria where they are thought to

inhibit the formation of permeability transition pores in the mitochondrial membranes, the net effect of this being reduced cell apoptosis during the first minutes of reperfusion following an ischaemic event (43).

### 7.3.2 RIPC and Endothelial Function

Translating results from animal model studies has proved difficult, with some studies, including meta-analyses, reporting a beneficial effect of trials of RIPC but also other large randomised controlled trials (RCT) which report no significant effect of RIPC. One of the first RCTs to report positive results was conducted by Cheung et al., who performed four five-minute occlusion / reperfusion cycles using an inflated blood pressure cuff around the lower limbs of children prior to them undergoing surgical repair of congenital heart defects. Compared to controls the RIPC group had significantly lower levels of post-operative Troponin I, indicating less myocardial injury post-operatively. The RIPC group also required significantly less inotropic support in the post-operative period(44). The Remote IMPACT RCT was an international, blinded, parallel-group RCT that measured the serum creatine kinase-myocardial band (CK-MB) after patients had undergone major cardiac surgery consisting of valvular repair, coronary artery bypass grafting (CABG) or a combination of both. There was no significant difference in post-operative CK-MB levels between the RIPC and sham RIPC group(45). Looking into the baseline and operative characteristics of the patients the RIPC group had more comorbidities with greater numbers having had previous cardiac surgery (29 vs 21) and myocardial infarctions (41 vs 35). There were more combined CABG and valve repair operations in the RIPC group (45 vs 38). This would lead to longer operating times and likely affect post-op CK-MB. In another RCT looking at RIPC and its effect on post-operative acute kidney injury (AKI) after cardiac surgery the authors(46) reported a significant reduction in AKI (37.5% vs 52.5%, 95% CI, 2.56% to 27.44%; P=0.02). They also reported that fewer patients required renal replacement therapy post-operatively (5.8% vs 15.8%, 95% CI 2,25% to 17.75%; P=0.01). There is mixed reporting for the benefit of RIPC in the post-operative setting. One of the noted differences between trials reporting significant results and those not, is the type of anaesthetic agent used. It has been suggested that Propofol based anaesthetic regimes could explain the lack of significant results observed. Propofol has been shown to abrogate the effects of RIPC in rats who have had a Propofol

anaesthesia as opposed to those who have had pentobarbitol or sevoflurane(47). It may be that preconditioning participants immediately prior to surgery is not an effective way to measure the effects of RIPC accurately if the participant is to have a Propofol based anaesthetic.

Whilst a lot of research has examined the effects of RIPC in the setting of cardiothoracic surgery there is growing evidence that RIPC can improve endothelial dysfunction. RIPC can increase circulating levels of eNOS which is responsible for most of the vascular nitric oxide produced(48). RIPC can reduce endothelial dysfunction as measured by FMD. Participants were given RIPC immediately before an ischaemic insult or at 4hrs, 24hrs and 48hrs before ischaemic insult. When RIPC is given immediately before an ischaemic insult it preserves endothelial function (FMD 9.4 +/- 0.7% vs 8.0 +/- 0.8%). Similarly when RIPC is delivered 24hrs and 48hrs before ischaemic insult endothelial function is preserved but not when the insult is delivered 4hrs after RIPC. This would suggest that there is an early and late phase of protection against endothelial injury(49). Most research has employed single bouts of RIPC whereas few have examined the effects of repeated daily cycles of RIPC. However, a trial in healthy volunteers who took part in a seven day trial of RIPC has previously been conducted. Participants had four five-minute occlusion / reperfusion cycles on the upper limb for seven consecutive days. The results showed that FMD increased after seven days of RIPC (5.0% +/- 2.2% and 6.1% +/- 2.2%, P=0.03). Interestingly when FMD was measured in the contralateral arm, FMD had increased suggesting that the protective effects of RIPC are conferred to distant organs and tissues(50).

Inducing RIPC by cycles of carotid artery occlusion and reperfusion in mice models has shown a significant decrease in cerebral infarct size after sustained occlusion of the middle cerebral artery(51). Furthermore the study also demonstrated increased immunoreactivity of nitric oxide synthase (NOS) in the cerebral vasculature. The effects of RIPC are neutralised in endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) null mice(52). RIPC can affect cytokine and chemokine expression in inflammatory pathways. It has been shown to decrease pro-inflammatory cytokines interleukin-1 (IL-1) and interleukin-6 (IL-6) expression and upregulates expression of anti-inflammatory cytokines such as IL-10(53). Reproducing these results in clinical trials has produced conflicting results. In paediatric cardiac

surgery patients who were subjected to RIPC, there was no difference in levels of IL-6, IL-8, IL-10 or TNF-alpha compared to controls(44) however another similar study looked at cytokine levels in patients undergoing orthopaedic procedures. The difference in levels of IL-6, IL-8 and Malondialdehyde (a biomarker of oxidative stress) were significantly reduced in the RIPC group compared to controls. Zagidullin et al. (54) demonstrated an improvement in arterial stiffness and endothelial function, in patients with angina, after repeated bouts of RIPC. There is a lack of research examining the effects of RIPC on endothelial function in participants with IC. At the time of writing no major trials had been completed specifically looking at the effects of RIPC in claudicants.

## 7.4 ASSESSMENT OF MICROVASCULAR FUNCTION

### 7.4.1 Laser Doppler Imaging with Iontophoresis

Iontophoresis is a well established method of transdermal delivery of small quantities of a drug using small electric currents. A Perspex chamber is placed on the skin, most commonly the forearm, and drug of choice is placed inside the chamber. A laser imager is then placed over the drug chamber to measure the effect of iontophoresis of the drug being iontophoresed.

**Iontophoresis**

The guiding principle is that molecules of a drug in solution that are either positively or negatively charged will migrate across the skin when subjected to a monopolar current(55). A positively charged chamber, the anode, will repel a positively charged chemical into the skin, likewise, a negatively charged chamber, the cathode, will repel a negatively charged chemical into the skin (Figure 3). A reference electrode is connected to an iontophoresis controller, and, to the participant being assessed to complete the circuit. The polarity of the reference electrode is altered based on the charge of the chemical being iontophoresed i.e a positively charged drug will have a negatively charged reference electrode and vice-versa. The amount of drug that passes through the skin will depend on

- Current – (The greater the current the more the like charges will repel each other)
- Time – (The longer the current is sustained the greater the amount of drug absorbed)
- Skin Resistance (The greater the resistance the lower the concentration of drug that will be absorbed)

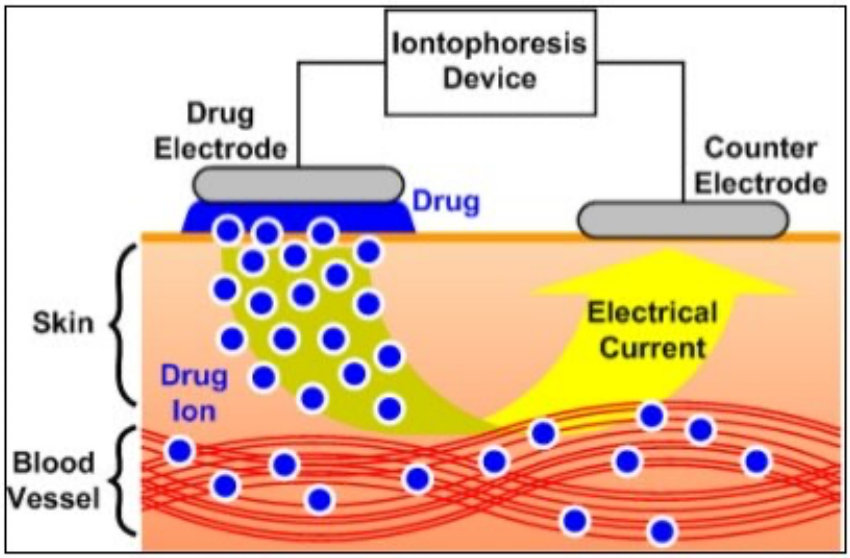



**Figure 3:** Diagrammatic representation of concept of iontophoresis

It is thought that ACh facilitates vasodilation during iontophoresis via endothelial dependent production of NO (Figure 4) with a possible supporting role played by endothelial-derived hyperpolarizing factor (EDHF)(56). Sodium Nitroprusside mediates vasodilation independently of the endothelium. SNP reacts with physiologic sulphydryl groups and stimulates production of NO directly(57).

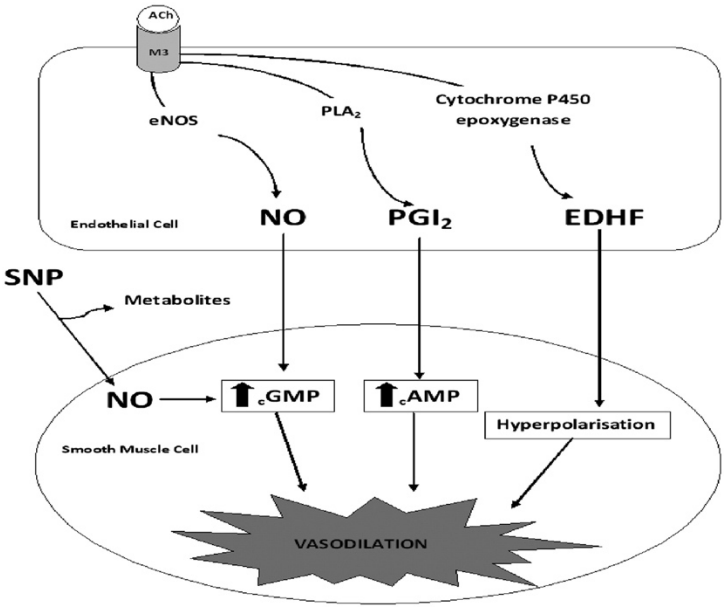



**Figure 4:** Role of ACh and SNP in vasodilation. Adapted from Turner et. al 2008.(56)

## Laser Doppler Flowmetry & Laser Doppler Imaging

The increased blow flow as a result of chemically induced vasodilation can be measured using Laser Doppler Flowmetry (LDF) or Laser Doppler Imaging (LDI). LDF was first used in this setting but it was limited in that when scanning a drug chamber it could only create a single point measurement on the skin whereas LDI could scan the area over the entire distribution of the drug chamber allowing a more detailed perfusion map. The LDI apparatus generally consists of a coherent light source which is usually a monochromatic laser with a long coherence length, a fast detector and a software unit for recording and analysing the detecting signal(58). The LDI detects moving red blood cells and static tissue. Moving red blood cells undergo a shift in frequency proportional to their velocity according to the Doppler principle(56). The difference in moving red blood cells and static tissue allows the LDI to build up a measurement of 'flux' which is an estimate of the blood flow. This 'flux' is expressed in arbitrary units called Perfusion Units (PU).

It has been shown that human cutaneous microvasculature can be used as a surrogate marker for systemic microvascular function(59). Khan et. al(60) demonstrated that assessment of cutaneous blood flow response to iontophoresis of ACh and SNP showed a positive, statistically significant, correlation with vasodilatory

response to adenosine within the Left Anterior Descending coronary artery suggesting that cutaneous blood flow is representative of the coronary circulation. In a study comparing young Type I diabetic patients with no clinically evident micro or macrovascular complications, with healthy controls, a reduced response to iontophoresis of ACh and SNP was found to be statistically significant ($p < 0.001$, for both ACh & SNP)(61). Furthermore reduced vasodilatory response to ACh, which is endothelial dependent, was significantly correlated with duration of diabetes and poor glycaemic control ($p < 0.001$).

Iontophoresis and LDI have their advantages in that it is a non-invasive assessment method, and it has been shown that changes of the microvasculature are representative of the systemic vasculature. It allows drugs to be introduced into the circulation without any needling or direct infusion into vessels. The amount of drug that needs to be used is very small and as these small quantities are absorbed trans-dermally it avoids first-pass metabolism. However, there can be complications arising from the procedure. Although rare it is recognised that side-effects such as skin irritation or burn can occur. This generally resolves over time but needs to be relayed to any potential study participants. Results can be affected by room temperature, recent ingestion of food or drink (tea, coffee) or recent strenuous exercise. These factors need to be controlled for to ensure accurate results especially if participants are attending over multiple visits. Variations in skin resistance across different anatomical sites of the same individual, and across individuals themselves, can affect the concentration of drug that passes to the microcirculation. It is thought that certain skin areas or individuals will have different levels of hair follicles and sweat ducts, which act as paths of least resistance for iontophoresed drugs(62)

## 7.5 ASSESSMENT OF MACROVASCULAR FUNCTION

### 7.5.1 Pulse Wave Analysis

Whilst LDI analyses endothelial function within a local vascular bed pulse wave analysis (PWA) is a non-invasive way of measuring global endothelial function(63). Arterial stiffness is partly dependent on vasomotor tone which itself is partly dependent on a functioning endothelium. With each heartbeat an arterial waveform moves

centrally through the arterial tree to the peripheral arteries. This waveform can be assessed using a highly sensitive applanation tonometer placed over the radial artery. The arterial pulse waveform is reflected back to the central circulation and this may alter the measured radial waveform. To account for this the computer software uses the Augmentation Index (AIx) which is a function of the relationship between the reflected arterial wave and the primary aortic wave(63). The AIx represents central aortic stiffness (Figure 5) and has been used as a surrogate marker for endothelial function(64).

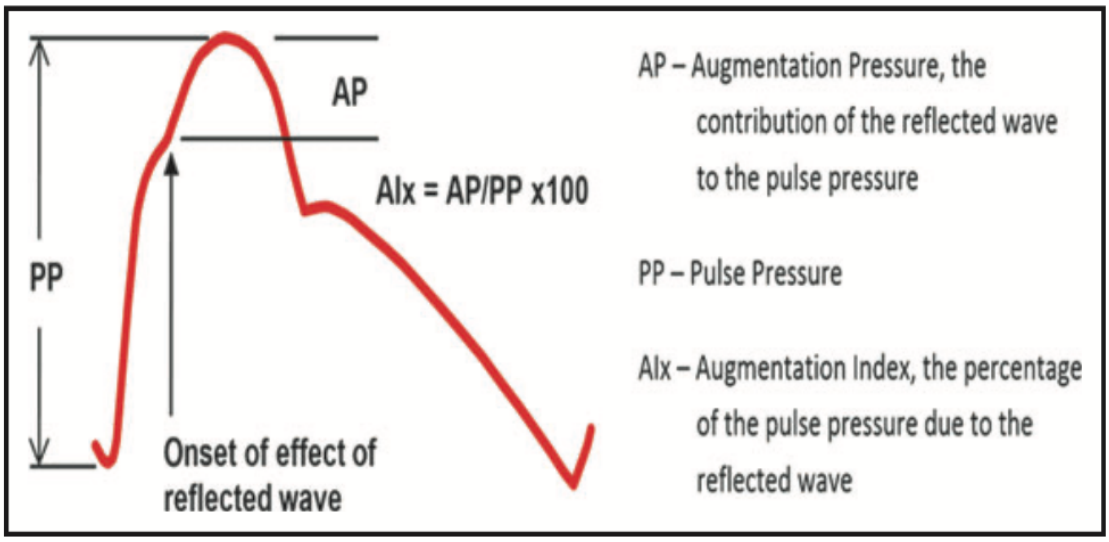



**Figure 5:** Aortic Pulse Wave. Adapted from Townsend et. al (2015)(65)

Central arterial stiffness is lower than in peripheral vessels in younger healthy individuals. In patients with worsening endothelial dysfunction or atherosclerotic disease central arterial stiffness increases and this alters the reflected wave observed. The reflected wave is usually observed during late systole but due to increased arterial stiffness it occurs during early systole (Figure 6).

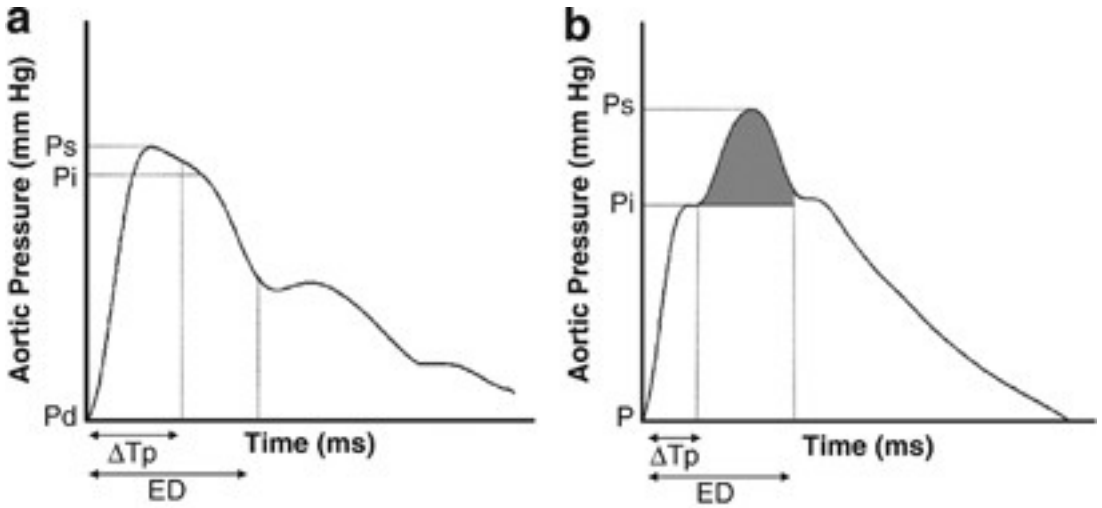



**Figure 6: "**Aortic pulse wave analysis. (**a**) Normal/young pulse waveform. Reflected wave during late systole, that is, longer Δ*T*p and Pi occurs after Ps which does not increase left ventricular effort. (**b**) Altered/old pulse waveform. Reflected wave during early systole produces an augmented systolic pressure, which decreases blood flow (not shown), and enhances a wasted ventricular effort (dashed area). Ps, systolic pressure; Pi, incident pressure from reflecting pressure wave; Pd, diastolic pressure; Δ*T*p, round trip travel time of the reflecting pressure wave; ED, ejection duration. Aortic augmentation index (AIx)=((Ps−Pi)/(Ps−Pd)) × 100". Adapted from Gurovich et. al (2011)(66)

### 7.5.2 Pulse Wave Velocity

Pulse wave velocity (PWV) is a gold standard for assessing arterial stiffness and it has been associated with cardiovascular mortality with an increase in arterial stiffness being associated with worse outcome(67). Pulse wave velocity is calculated by using a applanation tonometer to measure pulse waves at two different arterial sites. The most accurate way is to measure carotid and femoral pulse waveforms. The pulse wave velocity is calculated by dividing the distance travelled by the time delay between the pressure upstroke at both sites. In young healthy individuals there is a difference in pulse wave velocities when looking at central vessels compared to peripheral vessels. In the proximal aorta the PWV has been measured at 4 – 6 m/s whilst in the more peripheral arteries it is 8 – 10 m/s(68). It is thought that this is because the proximal arteries are more viscoelastic compared to the more resistant peripheral arteries. A pulse wave will be propagated much more quickly through a stiffer more resistant artery than a viscoelastic one(69). In this way a decrease in PWV velocity is thought to be associated with improvement in endothelial function.

### 7.5.3 Post Occlusive Reactive Hyperaemia

Post Occlusive Reactive Hyperaemia (PORH) is best achieved by placing a blood pressure cuff around the upper or lower limb and inflating it to a pressure that causes occlusion of the intended artery (Figure 7). The cuff remains inflated for set period of time after which it is quickly released and the resultant rapid increase in blood flow, or 'reactive hyperaemia', is measured.

**Figure 7:** Illustrative set-up for PORH using FLPI

This response involves various mediators that can alter myogenic tone including endothelium-derived hyperpolarising factor (EDHF), sympathetic nervous innervation of the microcirculation and the endothelial NO dependent pathway, although the latter plays a very small role(59). On release of the cuff the increased blood flow causes shear stress which stimulates release of NO, which vasodilates, further increasing blood flow. The post occlusive change in blood flow is assessed by measuring changes in skin microcirculation. This is most commonly done with either LDI or Full Field Laser Perfusion Imager (FLPI). The advantage of FLPI in this setting is that it is able to capture real time images approximately four times faster than LDI. The disadvantages of FLPI in this setting is that it is susceptible to movement artifact and participants must remain very still which can be difficult to achieve when a tight blood pressure cuff is inflated around one of their limbs. FLPI will be used to assess PORH

in the current study. To perform a PORH test there is a resting period, an occlusive period and a reperfusion period. During all three phases blood flow is being monitored. At the occlusion phase a biological zero is achieved. Despite the arterial occlusion the imager will still detect blood flow and it has been suggested that this could be due to Brownian movement within the interstitium(56). The biological zero should be considered when calculating results. Some advocate that the biological zero should be subtracted, others not. It appears that whichever method is used the end results of the study are unaffected(56)

# 8 AIMS & OBJECTIVES

**Primary Objective:**

To investigate the relationship between markers of vascular function (endothelial function, post-occlusive reactive hyperaemia & arterial stiffness) and repeated cycles of remote ischaemic preconditioning. The study will aim to demonstrate an improvement from baseline of microvasculature response to iontophoresis of ACh and SNP, PORH and arterial stiffness as measured by Pulse Wave Analysis & Pulse Wave Velocity.

**$H_0$ –** Repeated cycles of remote ischaemic preconditioning will have no effect on vascular function

# 9 METHODS AND MATERIALS

## 9.1 SPONSORSHIP AND ETHICAL APPROVAL

Sponsorship and R&D approval was gained locally from the Tayside Medical Science Centre and was granted on 19th December 2017.

Ethical approval was sought from the London – Westminster Research Ethics Committee. They confirmed that the study would be eligible for a proportionate review and full ethical approval for the study to commence was given on 6th February 2018.

## 9.2 SUBJECTS

Seventeen participants in total were recruited to the study. They consisted of nine healthy volunteers and eight participants with a clinical diagnosis of intermittent claudication. All participants were recruited to the study between 7th February 2018 to 9th October 2019. Written informed consent was obtained for each patient prior to participation in the study. All subjects had an initial consultation to discuss what was involved in participating in the study and to give them time to ask any questions they had before signing the consent form. Participants attended the blood flow laboratory on two separate occasions with each consultation lasting approximately 90 minutes. In between each consultation participants were given a manual sphygmomanometer and shown how to complete the Remote Ischaemic Pre-conditioning protocol at home. The protocol was completed over 4 consecutive days. Microvascular and macrovascular assessments were performed before commencing the protocol and 24 hours after finishing the RIPC protocol. On each visit participants had 40 mls of venous blood taken from the antecubital fossa of either the right or left arm to assess for markers of cardiovascular risk and were assessed using Iontophoresis, Pulse Wave Analysis & Velocity and Post-occlusive Reactive Hyperaemia (Table 1).

| | **Screening** | **First Visit** *(All patients)* | **Week 1** *(All patients)* | **Final Visit** *(All patients)* |
|---|---|---|---|---|
| **Consent** | √ | | | |
| **Blood samples for cytokine analysis** | | √ | | √ |
| **Iontophoresis & PWA/PWV** | | √ | | √ |
| **Remote Ischaemic Preconditioning** | | | √ | |

**Table 1:** Study matrix showing chronological order of study investigations and interventions for each participant

On the first consultation each participant was shown how to use the manual sphygmomanometer so that they could perform the remote ischaemic preconditioning protocol at home. The temperature of the Lab room was kept at 23 °C and patients were given 10 minutes to acclimatise before formal assessment started. Participants were asked to not smoke, eat any food or take on any fluids for at least 2 hours before attending the Lab.

### 9.2.1 Inclusion & Exclusion Criteria

Inclusion and exclusion criteria are detailed below:

**Inclusion Criteria:**

- Age 18 years and over
- Able to give written informed consent

**Exclusion Criteria:**

- Positive medical history of upper limb Deep Vein Thrombosis (DVT), Raynaud's disease or Sickle Cell Disease

- Previous medical history of upper limb DVT
- If patient is taking Glibenclamide or Nicorandil
- If participant has any upper limb fistula
- Unable to give written informed consent
- Previous arterial surgery
- Any contraindication to acetylcholine
- Any contraindication to sodium nitroprusside
- Any contraindication to blood pressure cuff inflation

## 9.3 REMOTE ISCHAEMIC PRECONDITIONING

### 9.3.1 Inducing Ischaemia

Ischaemic preconditioning was induced by placing the cuff of a standard manual sphygmomanometer over the biceps of the participant's arm. An initial blood pressure reading was taken to establish a baseline. The cuff was then inflated to a pressure 20mmHg greater than baseline and the wrist was assessed to ensure there was no palpable radial pulse. The cuff remained inflated at the desired pressure for 5 minutes, after which it was deflated, and the arm allowed to re-perfuse for 5 minutes. This counted as one cycle. In total four cycles were performed, consecutively, each day for four consecutive days. Patients were given a rudimentary diary card (Table 2) telling them what pressure to inflate the cuff to, and, also as an aide-memoir to help keep track of how many cycles they had performed. Participants were told to use the blood pressure cuff on the same arm each time they performed the RIPC protocol.

| | **1st Cycle** Inflate 5 mins Deflate 5 mins | **2nd Cycle** Inflate 5 mins Deflate 5 mins | **3rd Cycle** Inflate 5 mins Deflate 5 mins | **4th Cycle** Inflate 5 mins Deflate 5 mins |
|---|---|---|---|---|
| **Day 1** | | | | |
| **Day 2** | | | | |
| **Day 3** | | | | |
| **Day 4** | | | | |

*Remember: One cycle means inflating the blood pressure cuff for 5 minutes and then deflating it for 5 minutes. Do this four times and put an X in the box when you have done one cycle.*

**Table 2:** Diary Card provided to participants to help with completing the trial protocol.

## 9.4 ASSESSMENT OF MACROVASCULAR FUNCTION

### 9.4.1 Assessment of Arterial Stiffness

**Pulse Wave Analysis / Pulse Wave Velocity**

Patients were asked to lie supine on a bed for 10 minutes before performing the assessment. Pulse wave analysis and pulse wave velocity were measured using the cuff based SphygmoCor Xcel (AtCor Medical) device. To measure pulse wave analysis the cuff was placed around the upper arm over the normal anatomical lie of the brachial artery. The brachial artery waveforms were calibrated using cuff measured brachial systolic and diastolic pressures. The system uses a proprietary digital signalling and transfer function to generate central aortic pressure waveforms. The central aortic pulse wave is used to determine AIx. The system also normalises the AIx to a heart rate of 75 bpm (AIx@75) as AIx is affected by extremes of heart rate(70).

To measure pulse wave velocity the cuff was placed around the leg of the participant midway between the knee and hip. Three measurements were taken using a non-flex measuring tape. Distance was measured from the:

1. Sternal notch to the carotid pulse
2. Sternal notch to the proximal edge of the thigh cuff
3. Femoral artery at the midpoint of the inguinal ligament to the proximal edge of the cuff

A PWV value was formulated by calculating the ratio of the corrected distance between the carotid and femoral artery pulse measuring sites to the time delay between the two pulse waves.

## 9.5 ASSESSMENT OF MICROVASCULAR FUNCTION

### 9.5.1 Laser Doppler Imaging

All participants were assessed using LDI (Moor Instruments Ltd., Axminster, UK) iontophoresis of acetylcholine and sodium nitroprusside. Participants were asked to lie on a bed in the supine position with arms and legs placed in a comfortable position. LDI and iontophoresis of ACh and SNP were carried out on the lower legs of the participants and the same leg was assessed in each visit. For patients with claudication the symptomatic leg was used for each visit. The response to each drug was measured on the skin surface of the anterior compartment of the lower leg. The skin was prepared by using surgical tape to remove any loose debris on the skin. A chlorhexidine wipe was used to clean the area and then allowed to dry. The iontophoresis chamber (Moor Instruments Ltd., Axminster, UK) is a Perspex ring, measuring 20mm in diameter. A wire electrode penetrates the Perspex ring and inserts into the inner surface. A double-sided adhesive tape matched to the shape of the ring is attached to the bottom of the ring with the chamber then fixed to the anterior compartment of the lower leg. ACh (Sigma-Aldrich, St. Louis, MO, USA) and SNP (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in de-ionised water and made up to a concentration of 10 g/L (1%). 2mls of this solution were inserted into the chamber using a plastic syringe. For ACh the positive lead of a current source was connected to the chamber and a negative lead, to act as a reference electrode, was attached to a hydro-gel pad which was then placed on the leg 10cm distal to the drug chamber.

For iontophoresis of SNP the leads were swapped around. A 2mW helium-neon laser was positioned over the leg and focused on the area to be scanned. The area to be scanned was mapped out prior to commencing iontophoresis. The laser was positioned and calibrated to be 30cm away from the skin surface. The laser and its entire metal frame were locked in place to ensure the same area of skin was scanned each time (Figure 8).

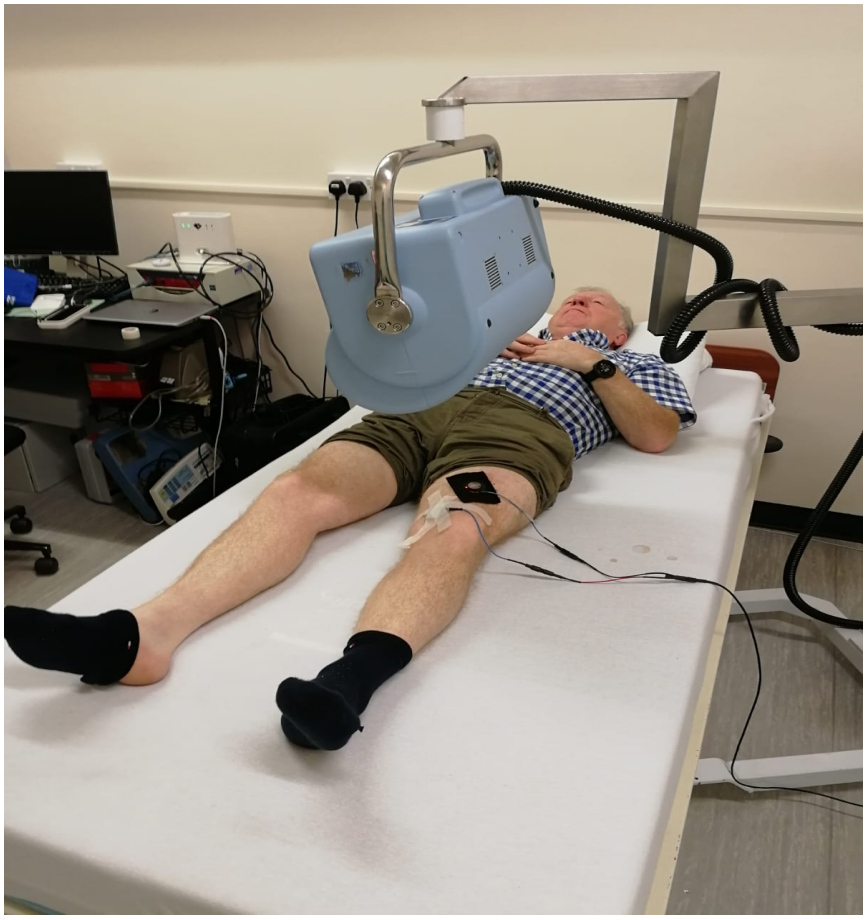


**Figure 8:** Illustrative set-up of how to perform iontophoresis on a leg

The LDI scans the selected area of the drug chamber for 60 seconds and will continue to re-scan the same area according to the protocol being used. Each scan creates a colour coded image representing skin blood perfusion over the targeted area. ACh and SNP both cause vasodilatation. ACh causes vasodilatation via endothelial dependent production of NO. SNP produces nitric oxide directly via reaction with tissue sulphydrl groups. The LDI measures back scattered light from moving erythrocytes. As vasodilation causes increased blood flow the frequency of the light returning to the laser is increased and this change is measured in Perfusion Units (PU).

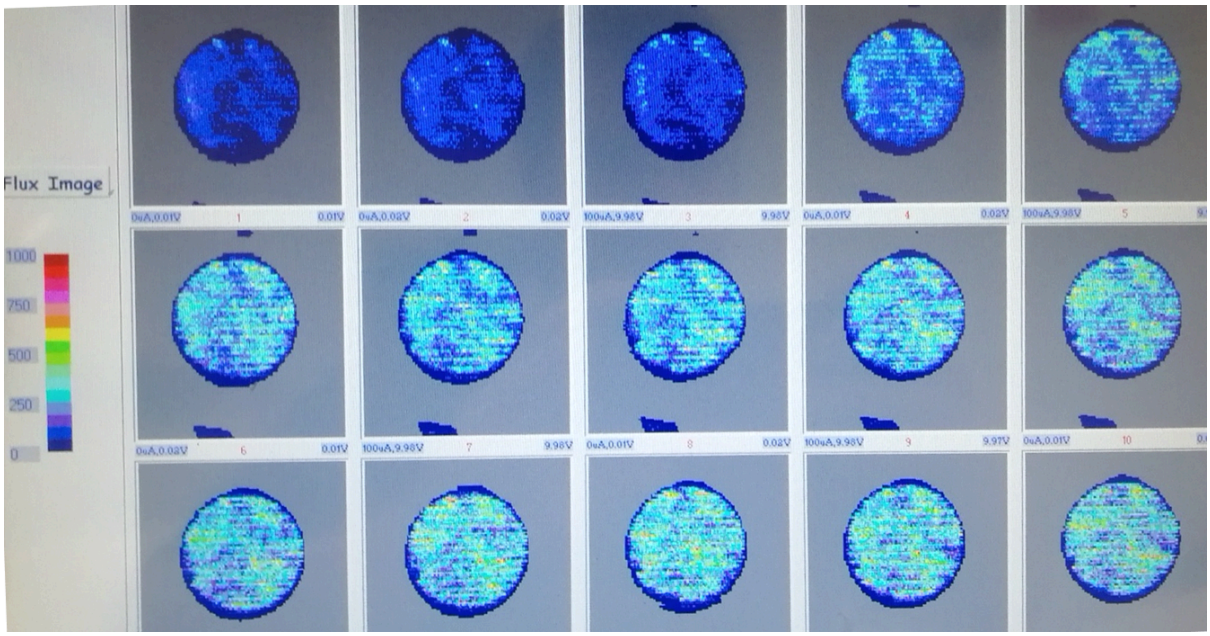



**Figure 9:** Scanned images for iontophoresis

When a current is passed through the chamber, the drugs inside iontophorese through the skin and get absorbed into the capillaries. Repeatedly passing a current through the chamber at regular intervals allows for the concentration to build up which increases vasodilation. A current of 100μA was passed through the chamber for 60 seconds followed by 60 seconds of no current. This was performed 6 times in total to produce twelve images. There were two baseline scans performed before this, and, at the end a further scan was performed to ensure the drugs had reached maximum concentration. This produced a total of 15 images (Figure 9). The protocol for iontophoresis was the same for ACh as for SNP.

### 9.5.2 Post Occlusive Reactive Hyperaemia

Patients were asked to lie on a bed and acclimatise to the room for 10 minutes. A manual blood pressure cuff was placed around the calf muscle of each participant. The same leg was used for each assessment. For participants with claudication the symptomatic leg was used for each assessment. Skin microcirculation was measured over the anterior compartment of the lower leg, below the level at which the blood pressure cuff was placed, using a full field laser perfusion imager (MoorFLPI, Moor Instruments Ltd., Axminster, UK). The low-power laser beam and a video frame of 10

images per second of blood perfusion of microvasculature of the skin was generated. A baseline measurement of skin perfusion was measured for 1 minute at which point the blood pressure cuff was inflated to a systolic blood pressure of 200mmHg occluding blood flow beyond it. None of the participants involved had an on-the-day measured blood pressure of greater than 180mmHg. The cuff remained inflated for a total of 5 minutes and then rapidly deflated resulting in a rapid return of blood to the lower leg. Post-occlusive blood flow was measured for a further 60 seconds. Peak perfusion minus biological zero was measured and used for analysis.

## 9.6 TISSUE ANALYSIS

### Tissue

In total around 80ml of blood was obtained from each participant over two visits with 40mls of blood being taken for each visit; 40ml for cytokine analysis and 40ml for markers of metabolic and oxidative stress.

Unfortunately due to onset of the coronavirus pandemic, and, altered working conditions for university and laboratory staff blood samples were not able to be analysed in time to be included in the finished study. The samples will be stored so that they may be used in further research and their implications for future research detailed in the discussion section.

## 9.7 STATISTICAL ANALYSIS

Baseline characteristics between the healthy volunteers and intermittent claudicants were compared using Mann Whitney tests for continuous variables and Fisher's exact tests for categorical data (due to the small sample size). Baseline iontophoresis, PWA, PWV and PORH values were compared between healthy volunteers and intermittent claudicants using Mann Whitney Tests.

Final iontophoresis, PWA, PWV and PORH values were also compared between healthy volunteers and intermittent claudicants using Mann Whitney Tests. Before and after RIPC values were compared using Wilcoxon Signed Rank tests for the group as a whole, and, healthy volunteers and intermittent claudicants separately. Scatterplots

and Spearman's correlations were used to identify associations between before RIPC treatment and after RIPC treatment PWA, PWV and PORH scores. Multiple regression models were created to identify any patient characteristics associated with significant differences in the final iontophoresis, PWA, PWV or PORH values.

All statistical analysis was performed using SPSS v26.

# 10 TRIAL OF PROTOCOL

As discussed in the introduction there is no general consensus in the literature as to what the optimum protocol for inducing RIPC is. Trials with similar protocols have reported conflicting results with statistically significant results found in participants who underwent one cycle of RIPC induction, and similar results in participants who underwent repeated cycles over many days, some as many as twenty-one consecutive days. There appears to more robust evidence that better results will be obtained by repeated cycles of RIPC over consecutive days and as such we performed limited protocol testing, using LDI iontophoresis, in five healthy volunteers to demonstrate proof of concept. Two participants performed 4 days of the RIPC protocol. Two participants extended the time frame to 7 days of RIPC and one participant performed 1 day of the RIPC protocol. All participants were tested before induction of RIPC and 24 hours after their last cycles of RIPC. For analysis purposes the median values of Perfusion Units (PU) were used as this was how it would be calculated in the formal trial. The baseline measurement was the lowest value of the first two scans and the peak scan was the highest value of the last two scans. The change in flux, as measured in Perfusion Units (PU), is the difference between the final scan and the baseline scan. The results for these and the difference in scores for before and after the RIPC intervention are notated (Table 3).

| | | ACh | | | SNP | | |
|---|---|---|---|---|---|---|---|
| **Participant No.** | **Number of days of RIPC** | **Before RIPC (PU)** | **After RIPC (PU)** | **Change in PU from baseline** | **Before RIPC (PU)** | **After RIPC (PU)** | **Change in PU from baseline** |
| **1** | **1** | 191 | 142 | -49 | 102 | 106 | 4 |
| **2** | **4** | 184 | 192 | 8 | 182 | 133 | 49 |
| **3** | **4** | 128 | 180 | 52 | 189 | 191 | 2 |
| **4** | **7** | 147 | 396 | 249 | 134 | 177 | 43 |
| **5** | **7** | 236 | 620 | 384 | 201 | 188 | -13 |

**Table 3** Change in Perfusion Units (PU) before and after RIPC for ACh and SNP in trial of protocol. PU are arbitrary units that are used as a measure of change in blood flow in response to ACh / SNP

**Participant 1**

This participant was a healthy volunteer with no current or past medical history and was not taking any prescription medication. They were taken to our laboratory and all the same study protocols were followed as were intended for the main trial. This participant was assessed using LDI and iontophoresis of ACh and SNP over the anterior compartment of the lower leg. The participant then underwent one day of the RIPC protocol. This involved four consecutive cycles of five minutes occlusion / reperfusion only. The participant was then reassessed using LDI and iontophoresis 24 hours later. The response from baseline is shown for both ACh and SNP. As can be seen the post intervention change in flux was actually lower than baseline (Figure 10).

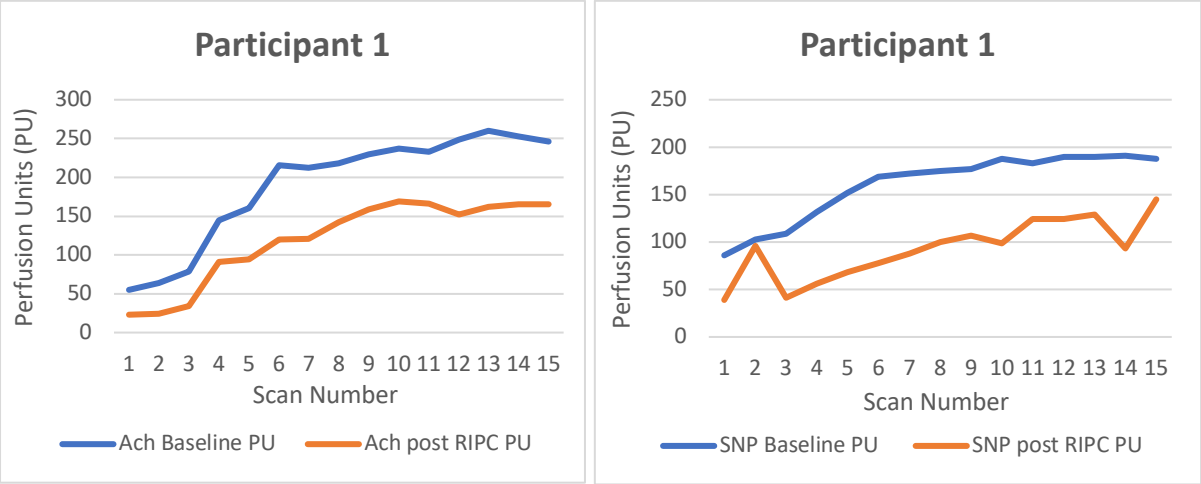



**Figure 10:** Trial of Protocol graph showing before and after values in Perfusion Units for ACh & SNP for participant 1 who had 1 day of RIPC protocol

**Participant 2 & 3**

These participants were healthy volunteers with no current or past medical histories and were not taking any prescription medication. They were taken to our laboratory and all the same study protocols were followed as were intended for the main trial. These participants were assessed using LDI and iontophoresis of ACh and SNP over the anterior compartment of the lower leg. The participants then underwent 4 days of the RIPC protocol. This involved four consecutive cycles of five minutes occlusion /

reperfusion for 4 consecutive days. The participants were then reassessed using LDI and iontophoresis 24 hours after the last cycle of RIPC was performed. The response from baseline is shown for both ACh and SNP for participants 2 and 3 (Figure 11 & 12).

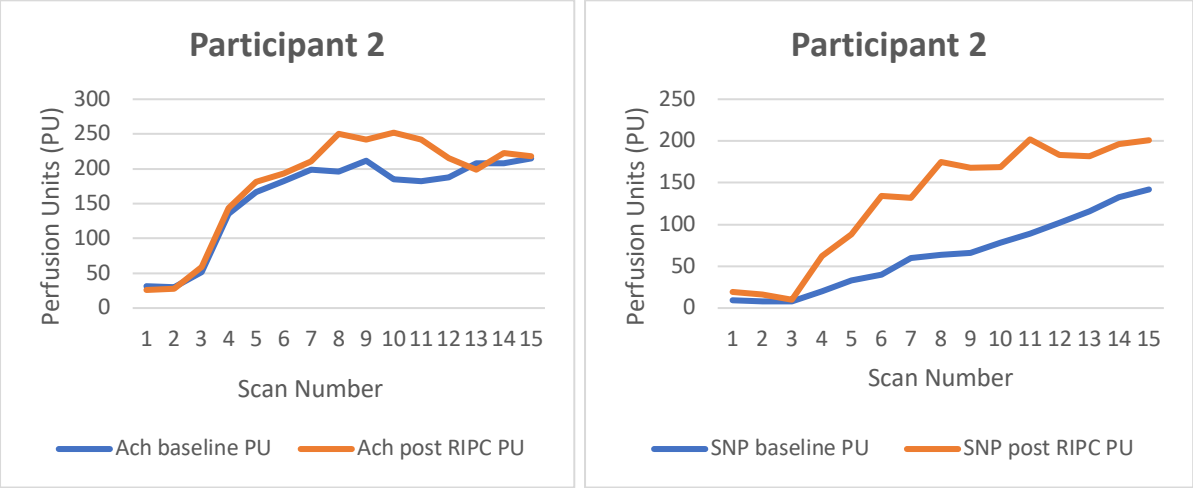



**Figure 11:** Trial of Protocol graph showing before and after values in Perfusion Units for ACh & SNP for participant 2 who had 4 day of RIPC protocol

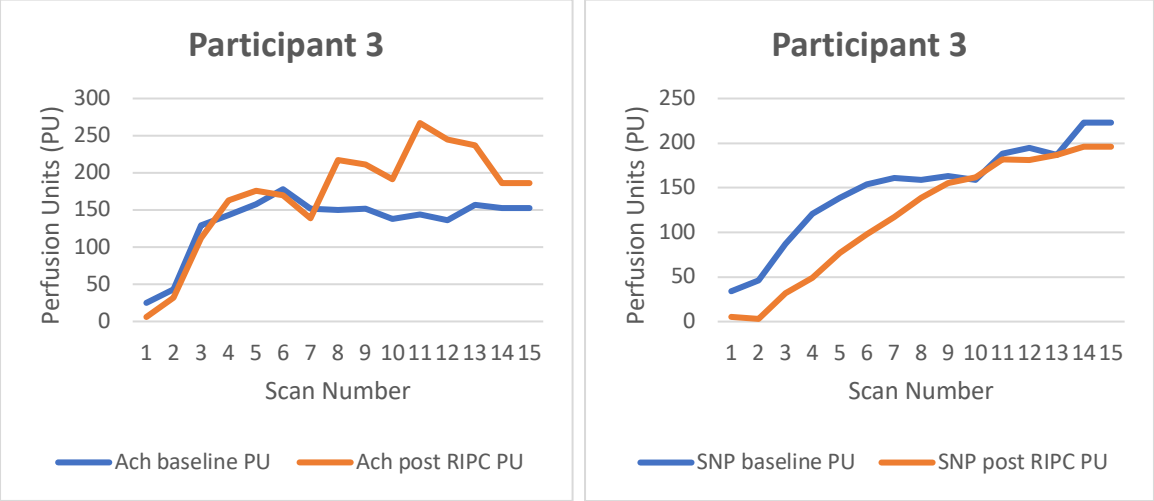



**Figure 12:** Trial of Protocol graph showing before and after values in Perfusion Units for ACh & SNP for participant 3 who had 4 day of RIPC protocol

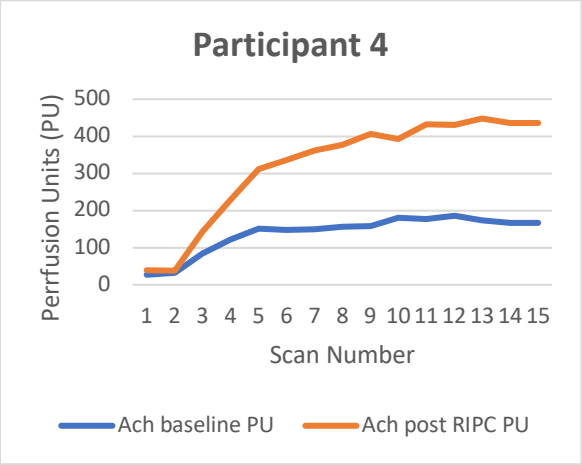
For participant 2 the RIPC protocol does not seem to have had much of an effect on ACh mediated vasodilation. For analysis purposes the median values of Perfusion Units (PU) were used as this was how it would be calculated in the formal trial. The

baseline measurement was the lowest value of the first two scans and the peak scan was the highest value of the last two scans. The change in flux, as measured in Perfusion Units (PU), is the difference between the final scan and the baseline scan. Baseline PU of 184 compared to 192 was observed. Looking at the overall trend in the graph there is a much greater peak in ACh post RIPC PU between scans 9 and 12 indicating a stronger response than perhaps the final numbers convey. We inspected the Perspex chamber at the end of the scanning process and although there was still small amount of ACh within the chamber there may not have been enough towards the end of scanning to cause further vasodilation beyond the peak seen between scans 9 and 12. Through the scanning process the drug chambers in some participants did need to be topped up with the drug being used. We found that if the drug chamber emptied it could produce a spurious increase in PU recorded. For this reason we chose to use the baseline and final scan PU for analysis as it was felt this would ensure the most accurate data was used.

Looking at the response to SNP in participant 3 the graph suggests the response to iontophoresis of SNP was reduced after four days of RIPC but the change in PU, when calculated as described above, were broadly similar. Baseline peak PU was 189 compared to an after RIPC peak PU of 191. Given that our hypothesis is that RIPC will improve endothelial function it was predicted that vasodilatory response to SNP, which is endothelium-independent, may not differ between pre-intervention and post-intervention scans.

**Participant 4 & 5**

These participants were healthy volunteers with no current or past medical histories and were not taking any prescription medication. They were taken to our laboratory and all the same study protocols were followed as were intended for the main trial. These participants were assessed using LDI and iontophoresis of ACh and SNP over the anterior compartment of the lower leg. The participants then underwent 7 days of the RIPC protocol. This involved four consecutive cycles of five minutes occlusion / reperfusion for 7 consecutive days. The participants were then reassessed using LDI and iontophoresis 24 hours after the last cycle of RIPC was performed. The response from baseline is shown for both ACh and SNP for participants 4 and 5 (Figure 13 & 14).



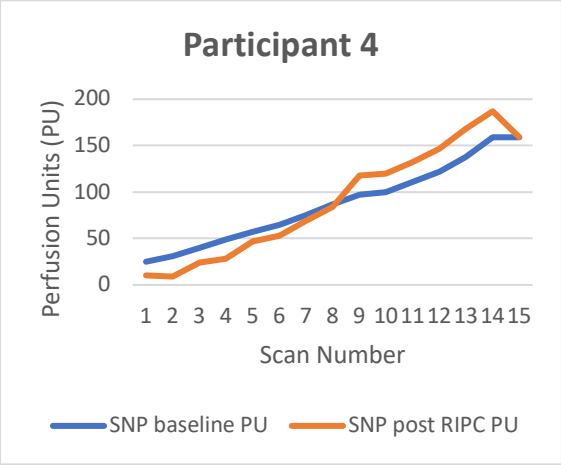
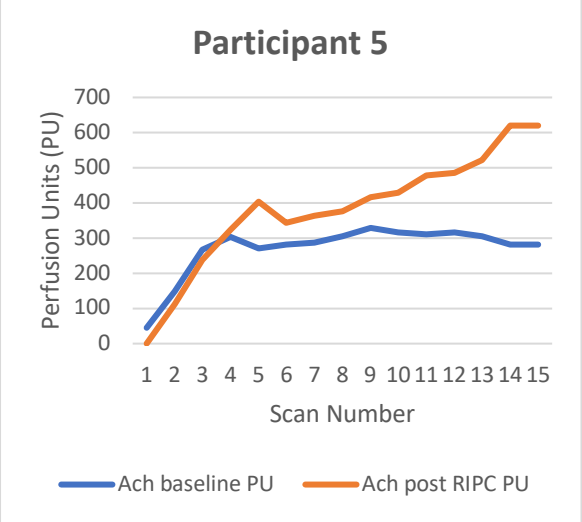




**Figure 13** Trial of Protocol graph showing before and after values in Perfusion Units for ACh & SNP for participant 4 who had 7 day of RIPC protocol



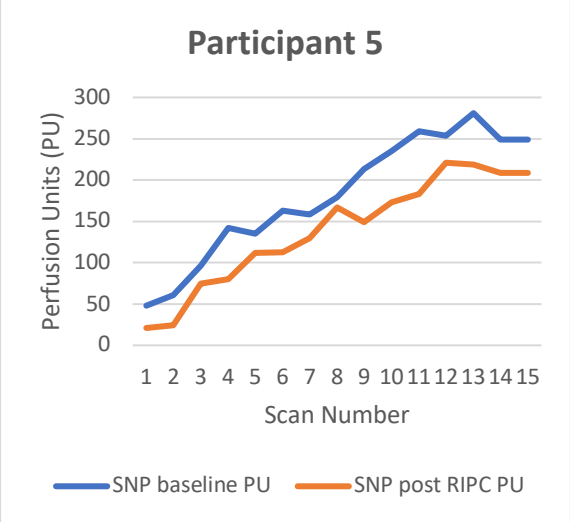




**Figure 14** Trial of Protocol graph showing before and after values, in Perfusion Units for ACh & SNP for participant 5 who had 7 day of RIPC protocol

Participants 4 and 5 displayed a much greater effect of RIPC after seven consecutive days and ideally we would have chosen to investigate the effects of a 7 day trial. Unfortunately the feedback from participants was that completing 7 days in total was very time consuming. Participants stated that they felt taking part in the trial was a major inconvenience. Data is presented for two participants but initially there were 5 participants. Three of the participants got in contact to communicate that they hadn't completed the full 7 days of RIPC. The concern was that, with 60% of the participants undergoing a 7 RIPC protocol not completing it fully, if we attempted to do this in the

main study we would have a high drop out rate, or, alternatively if patients did not drop out they may not have completed the full 7 days, and not admit this, which would weaken any effect size seen.

With this in mind we elected to use a 4 day RIPC protocol in the hope that we would avoid a high drop out rate and maintain compliance with the protocol. Although the trial of protocol numbers (N=5) were small it was to demonstrate proof of concept using iontophoresis for assessment. A positive response to the 4 day RIPC protocol was observed and this is what was used in the formal trial.

# 11 RESULTS

## 11.1 BASELINE CHARACTERISTICS

Among the seventeen participants eight had a diagnosis of intermittent claudication and nine were healthy volunteers. All the Participants with IC were smokers and none of the healthy volunteers smoked. Median age in the IC group was 60.0 years and was 40.0 years in the healthy volunteers which was a statistically significant difference (p=0.027). There were no significant differences in gender, systolic or diastolic blood pressure between the two groups (Table 4).

| | | **Intermittent Claudication** | **Healthy Volunteers** | **Statistical test** | **P value** |
|---|---|---|---|---|---|
| **Age**<br>Median *(IQR)* | | 60.0 *(14.5)* | 40.0 *(30.5)* | Mann-Whitney Test | 0.027 |
| **Sex** | Male<br>Female | 7<br>1 | 5<br>4 | Fisher's Exact Test | 0.294 |
| **Smoking status** | Smoker<br>Non-smoker | 8<br>0 | 0<br>9 | Fisher's Exact Test | <0.001 |
| **Systolic Blood Pressure (mmHg)**<br>Mean *(SD)* | | 129 *(11)* | 124 *(20.5)* | Mann-Whitney Test | 0.277 |
| **Diastolic Blood Pressure (mmHg)**<br>Mean *(SD)* | | 81.5 *(19.75)* | 80.0 *(13.5)* | Mann-Whitney Test | 0.541 |

**Table 4** Baseline characteristics of main study variables for whole study group (*n*=17)

## 11.2 BASELINE VALUES

### 11.2.1 Iontophoresis of Acetylcholine and Sodium Nitroprusside

Alternate repeated currents of 100μA were passed through the drug chamber as previously described. The resultant level of vasodilation, measured by change in flux or change in perfusion units, was measured by calculating the difference in peak PU from baseline PU. These scores were examined, for differences, between healthy volunteers and participants with claudication before any RIPC had been commenced. There were no significant differences in scores between groups either in response to iontophoresis of either ACh or SNP (Table 5).

| | **Intermittent Claudication** | **Healthy Volunteers** | **Statistical test** | **P value** |
|---|---|---|---|---|
| Flux Change in PU Before Treatment: Acetylcholine Iontophoresis *Median (IQR)* | 215.5 *(244.25)* | 215.0 *(127)* | Mann-Whitney Test | 1.000 |
| Flux Change in PU Before Treatment: SNP Iontophoresis *Median (IQR)* | 87.0 *(214.5)* | 140.0 *(131.0)* | Mann-Whitney Test | 0.423 |

**Table 5** Effect of intermittent claudication on baseline ACh dependent and independent (SNP) endothelial function as measured by laser doppler imaging. Mann-Whitney Tests of iontophoresis Perfusion Unit (PU) values with study group split into IC & HV

### 11.2.2 Pulse Wave Analysis / Pulse Wave Velocity

There were no significant differences in baseline scores for pulse wave velocity but analysis did highlight significant differences between participants with IC and healthy volunteers for baseline pulse wave analysis scores (Table 6).

Claudicants had a higher baseline median PWA of 40.0 compared to 11.0 in healthy volunteers ($p = 0.015$) highlighting a significant difference in arterial stiffness compared to HV.

| | Intermittent Claudication | Healthy Volunteers | Statistical test | P value |
|---|---|---|---|---|
| Baseline Pulse Wave Velocity (m/s) Median *(IQR)* | 9.5 *(2.72)* | 7.8 *(2.6)* | Mann-Whitney Test | 0.139 |
| Baseline Pulse Wave Analysis (AIx) Median *(IQR)* | 40.0 *(10.75)* | 11.0 *(35.0)* | Mann-Whitney Test | 0.015 |

**Table 6** Effect of intermittent claudication on baseline arterial stiffness as measured by PWA and PWV. Mann-Whitney of baseline PWA / PWV values comparing IC (n=8) & HV (n=9) groups. PWV is measured in metres per second (m/s). PWA is measured as Augmentation Index (AIx), as previously described.

### 11.2.3 Post Occlusive Reactive Hyperaemia

There were no significant differences between baseline PU values for participants with IC or HV (Table 7, N=13, p = 0.181).

| | Intermittent Claudication | Healthy Volunteers | Statistical test | P value |
|---|---|---|---|---|
| PORH PU Before RIPC Median *(IQR)* | 87.2 *(130.9)* | 167.1 *(126.7)* | Mann-Whitney Test | 0.181 |

**Table 7** Effect of intermittent claudication on baseline endothelial function as measured by FLPI. Mann-Whitney test of baseline values for PORH, measured in PU, with study group split into IC (n=7) & HV (n=6).

## 11.3 RESULTS AFTER RIPC

### 11.3.1 Iontophoresis of ACh and SNP

Examination of median PU values for before and after RIPC intervention did not reveal an obvious change in values in response to either ACh or SNP (Figure 15 & 16). In the iontophoresis results, a higher score for ACh would indicate an improvement in endothelial function.

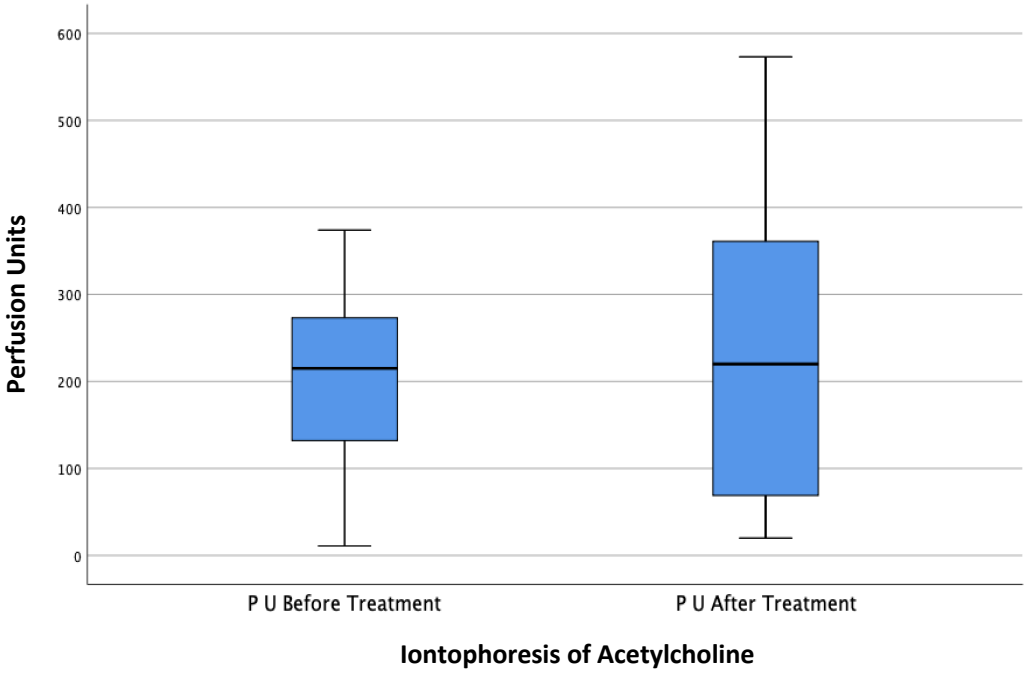



**Figure 15** Box and Whisker plot of change in ACh PU values after RIPC in whole study group (n=17). PU Before Treatment: Median = 215, IQR = 188.5; PU After Treatment: Median = 220, IQR = 313.

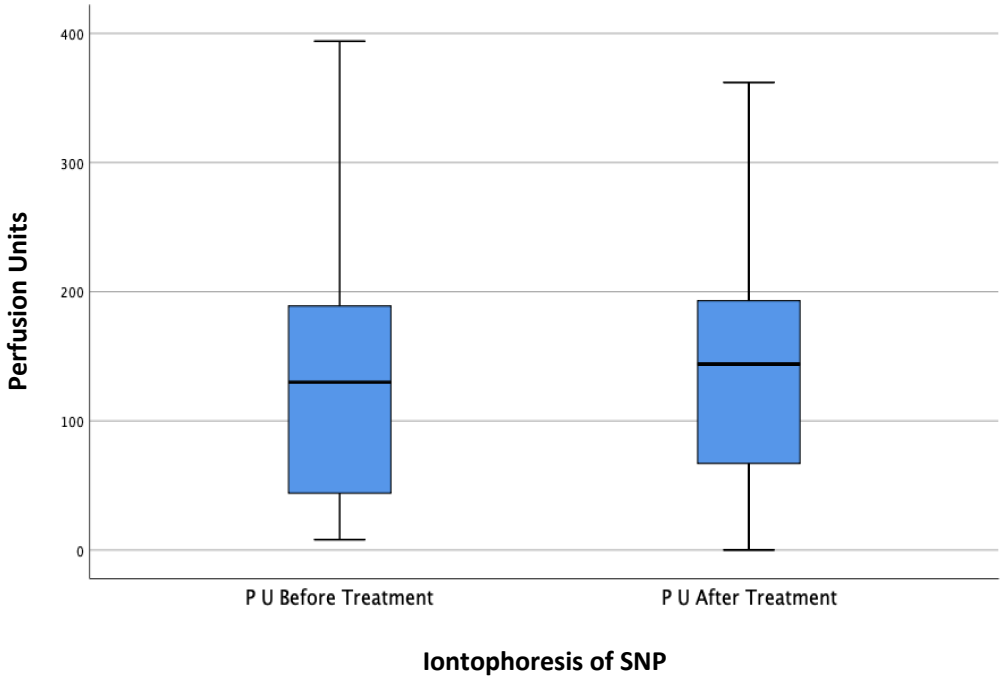



**Figure 16** Box and Whisker plot of change in SNP PU values after RIPC in whole study group (n=17). PU Before Treatment: Median = 130, IQR = 157; PU After Treatment: Median = 144, IQR = 142.5.

To look for any differences based on disease status the study sample was separated into IC and HV. Although the intervention appeared to have a more beneficial effect on healthy volunteers this did not reach statistical significance (Table 8). In response to ACh HV had a median increase of 20.0 units compared with 0.5 (p = 0.815). SNP mediated vasodilation was slightly less following RIPC for healthy volunteers compared with ACh.

| | **Intermittent Claudication** | **Healthy Volunteers** | **Statistical test** | **P value** |
|---|---|---|---|---|
| Effect of RIPC on Change in PU for ACh, Median *(IQR)* | 0.5*(440.5)* | 20.0 *(184.5)* | Mann-Whitney Test | 0.815 |
| Effect of RIPC on Change in PU for SNP, Median *(IQR)* | 10.0 *(205.75)* | 4.0 *(185.5)* | Mann-Whitney Test | 0.888 |

**Table 8** Effect of RIPC on ACh dependent and independent (SNP) endothelial function as measured by laser doppler imaging. Mann-Whitney Tests comparing ACh & SNP Perfusion Unit values between IC (n=8) & HV (n=9)

**Multiple Regression Analysis**

Linear regression analyses were performed to assess any variables associated with change in PU. No variables were significantly associated with change in PU. There appears to be a trend towards an association between diastolic blood pressure and change in PU, however, this did not reach statistical significance (Table 9, p = 0.078) in the ACh analysis but was significant in the SNP analysis (Table 10, p = 0.049). Looking at the analysis for change in ACh PU it seems that participants were more likely to have a lower change in PU if they had a diagnosis of IC although this was not significant (p = 0.944). There seems to be a different response to SNP according to gender as being female resulted in a greater change in PU after RIPC, although again this did not reach statistical significance (Table 10, p = 0.627). Interestingly diastolic blood pressure appears to lead to a greater change in PU, potentially suggesting that a higher diastolic BP results in greater ACh mediated vasodilation.

**Dependent Variable: Change in ACh PU**

| | Unstandardized Coefficients | | | | 95% Confidence Interval for B | |
|---|---|---|---|---|---|---|
| | Effect Estimate (B) | Standard Error | T statistic | P Value | Lower Bound | Upper Bound |
| IC Group | -10.366 | 143.856 | -0.072 | 0.944 | -330.898 | 310.165 |
| Age (year) | 2.118 | 5.064 | 0.418 | 0.685 | -9.166 | 13.401 |
| Female Sex | 58.693 | 109.761 | 0.535 | 0.605 | -185.870 | 303.257 |
| Diabetes | -124.471 | 162.025 | -0.768 | 0.460 | -485.486 | 236.544 |
| Systolic BP (mmHg) | -8.591 | 5.611 | -1.531 | 0.157 | -21.093 | 3.912 |
| Diastolic BP (mmHg) | 11.818 | 6.018 | 1.964 | 0.078 | -1.591 | 25.227 |
| (Constant) | 101.033 | 573.173 | 0.176 | 0.864 | -1176.075 | 1378.142 |

**Table 9** Multiple regression analysis to evaluate factors that impact ACh dependent endothelial function in response to RIPC, measured in PU values. N = 17

**Dependent Variable: Change in SNP PU**

| | Unstandardized Coefficients | | | | 95.0% Confidence Interval for B | |
|---|---|---|---|---|---|---|
| | Effect Estimate (B) | Standard Error | T Statistic | P Value | Lower Bound | Upper Bound |
| IC Group | -58.607 | 96.512 | -0.607 | 0.557 | -273.650 | 156.435 |
| Age (year) | 0.621 | 3.397 | 0.183 | 0.859 | -6.949 | 8.191 |
| Female Sex | -36.872 | 73.638 | -0.501 | 0.627 | -200.948 | 127.205 |
| Diabetes | -21.391 | 108.702 | -0.197 | 0.848 | -263.594 | 220.812 |
| Systolic BP (mmHg) | -3.294 | 3.765 | -0.875 | 0.402 | -11.682 | 5.095 |
| Diastolic BP (mmHg) | 9.020 | 4.037 | 2.234 | 0.049 | 0.024 | 18.016 |
| (Constant) | -268.451 | 384.538 | -0.698 | 0.501 | -1125.256 | 588.354 |

**Table 10** Multiple regression analysis to evaluate factors that impact ACh independent (SNP) endothelial function in response to RIPC, measured in PU values. N= 17

### 11.3.2 Pulse wave analysis

To see an improvement in endothelial function the PWA should decrease after the intervention.

## Spearman's Correlation

Spearman's correlation was performed to assess any association between before and after values for pulse wave analysis. Before and after scores were strongly correlated (Figure 17, Spearman's rho = 0.841, p = <0.001).

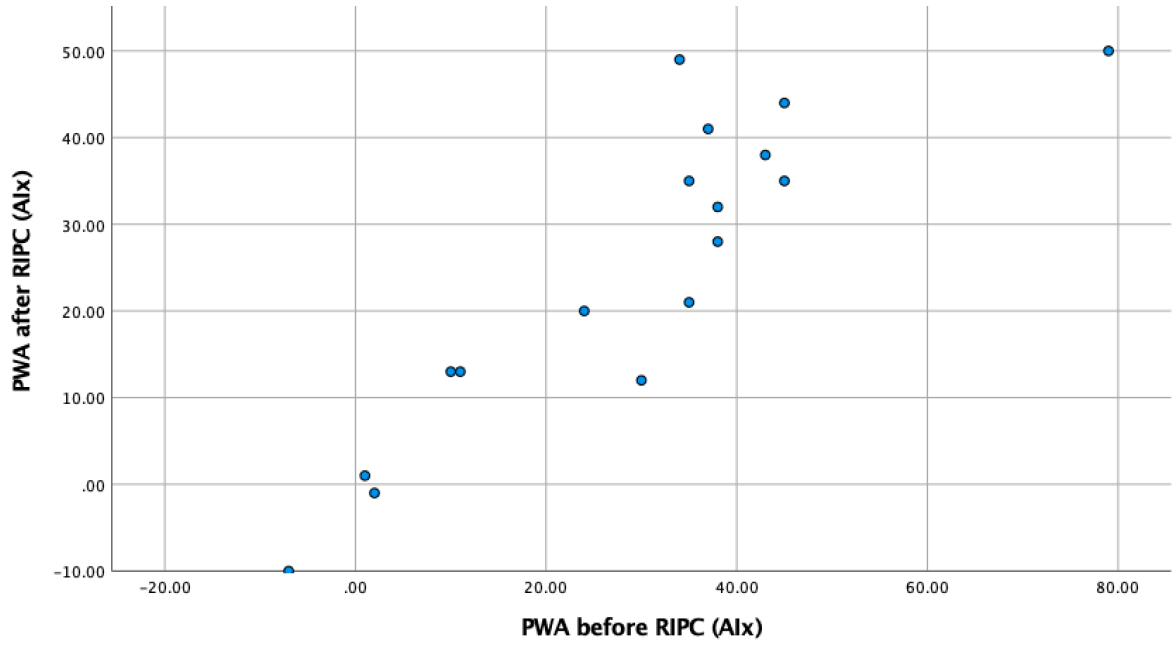



**Figure 17** Scatter plot looking at correlation of PWA values before and after RIPC across all study participants (*n*=17)

Looking at differences between IC and HV we see that there is a significant difference in pre intervention PWA values (p = 0.015) and also significant differences in post intervention values (p <0.001) between IC and HV (Table 11).

| | **Intermittent Claudication** | **Healthy Volunteers** | **Statistical test** | **P value** |
|---|---|---|---|---|
| Pre RIPC PWA (AIx) Median *(IQR)* | 40.0 *(10.75)* | 11.0 *(35.0)* | Mann-Whitney Test | 0.015 |
| Post RIPC PWA (AIx) Median *(IQR)* | 39.5 *(12.75)* | 13.0 *(24.5)* | Mann-Whitney Test | <0.001 |
| Change in PWA (AIx) After RIPC Median *(IQR)* | -2.5 (*11.75)* | -3.0 (13.0*)* | Mann-Whitney Test | 0.673 |

**Table 11** Comparison of effect of RIPC on arterial stiffness between IC and HV as measured by PWA (AIx). Mann-Whitney Tests evaluating differences in the pre-RIPC PWA values between IC and HV

groups, post-RIPC PWA values between IC and HV groups, and change in PWA values (post minus pre PWA values) between IC and HV groups

In order to assess whether being a healthy volunteer or participant with IC was predictive of post RIPC pulse wave analysis scores we preformed regression analysis controlling for potential confounders (Table 12).

**Multiple Regression Analysis PWA**

**Outcome Variable: Post Treatment PWA**

| | Unstandardized Coefficients Effect | | | | 95% Confidence Interval for B | |
|---|---|---|---|---|---|---|
| | Estimate (B) | Std. Error | T Statistic | P Value | Lower Bound | Upper Bound |
| Pre RIPC PWA | 0.536 | 0.239 | 2.240 | 0.052 | -0.005 | 1.078 |
| IC group | 14.337 | 6.339 | 2.262 | 0.050 | -0.003 | 28.677 |
| Age (year) | 0.022 | 0.358 | .062 | 0.952 | -0.789 | 0.833 |
| Systolic BP (mmHg) | -0.135 | 0.250 | -0.539 | 0.603 | -0.700 | 0.430 |
| Diastolic BP (mmHg) | 0.295 | 0.332 | 0.887 | 0.398 | -0.457 | 1.046 |
| diabetes | -4.626 | 7.408 | -0.624 | 0.548 | -21.384 | 12.132 |
| Female Sex | 1.436 | 4.904 | 0.293 | 0.776 | -9.657 | 12.529 |
| (Constant) | -4.531 | 31.177 | -0.145 | 0.888 | -75.060 | 65.997 |

**Table 12** Multiple regression analysis to evaluate factors that impact arterial stiffness in response to RIPC, as measured by PWA(AIx). N= 17

When controlling for potential confounders being a participant with IC was associated with a 14.337 point higher PWA score after undergoing the RIPC protocol when compared to healthy volunteers. This was statistically significant, p = 0.05.

### 11.3.3 Pulse Wave Velocity

To see an improvement in endothelial function the PWV should decrease after the intervention.

**Spearman's correlation**

Spearman's correlation was performed to assess any association between before and after values for pulse wave analysis. Before and after scores were strongly correlated (Figure 18).

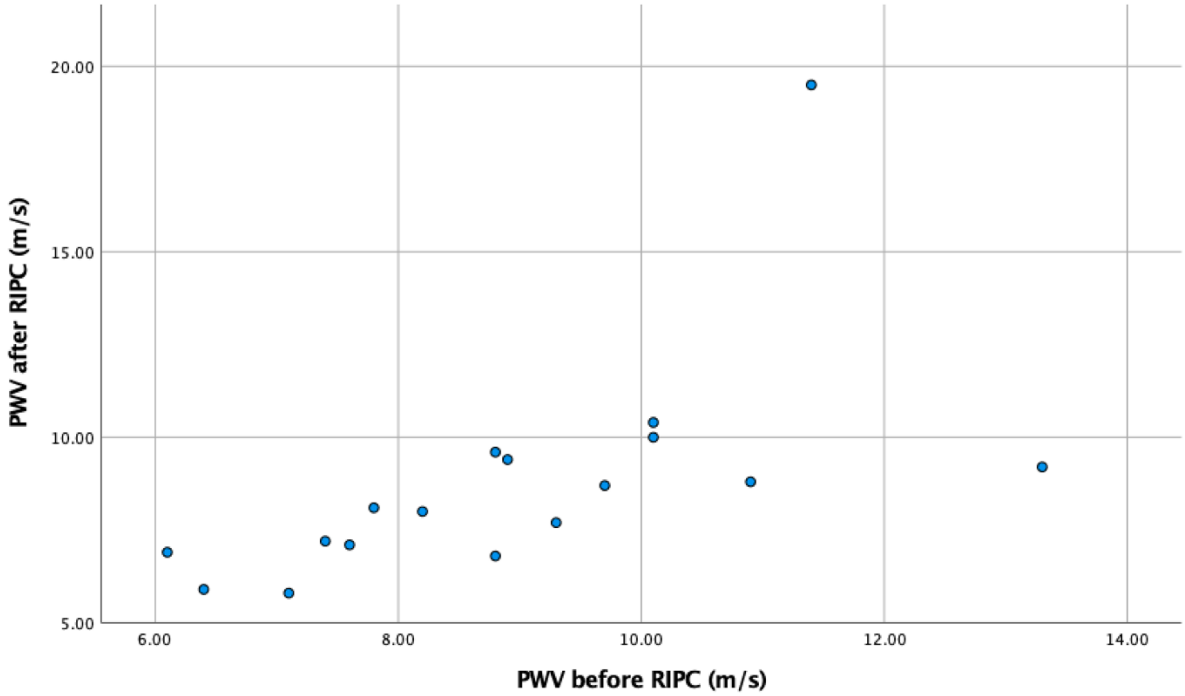



**Figure 18** Scatter plot looking at correlation of PWV values before and after RIPC (Spearman's rho = 0.795, p < 0.001, *n*=17)

**Mann-Whitney comparing PWV in IC and HV**

Healthy volunteers tended to have lower PWV values before and after the intervention compared to IC although this was not statistically significant (Table 13).

| | **Intermittent Claudication** | **Healthy Volunteers** | **Statistical test** | **P value** |
|---|---|---|---|---|
| Pre RIPC PWV (m/s) *Median (IQR)* | 9.5 *(2.72)* | 7.8 *(2.60)* | Mann-Whitney Test | 0.139 |
| Post RIPC PWV (m/s) *Median (IQR)* | 8.95 *(2.13)* | 7.2 *(2.25)* | Mann-Whitney Test | 0.277 |
| Change in PWV (m/s) After RIPC *Median (IQR)* | -0.60 *(2.1)* | -0.20 *(1.65)* | Mann-Whitney Test | 0.815 |

**Table 13** Comparison of effect of RIPC on arterial stiffness between IC and HV as measured by PWV. Mann-Whitney tests comparing PWV values, measured in metres per second, in IC and HV

HV tended to have lower PWV values post RIPC compared with before RIPC PWV values, however this was not statistically significant. Very little difference was observed in the PWV scores before and after RIPC for IC (Table 14).

| | **Before RIPC PWV (m/s)** | **After RIPC PWV (m/s)** | **Statistical test** | **P value** |
|---|---|---|---|---|
| All Patients *Median (IQR)* | 8.8 *(2.6)* | 8.1 *(2.5)* | Wilcoxon Signed Rank Test | 0.246 |
| Healthy Volunteers *Median (IQR)* | 7.8 *(2.60)* | 7.2 *(2.25)* | Wilcoxon Signed Rank Test | 0.513 |
| Intermittent Claudicants *Median (IQR)* | 9.5 *(2.72)* | 8.95 *(2.13)* | Wilcoxon Signed Rank Test | 0.327 |

**Table 14** Effect of RIPC on arterial stiffness as measured in PWV (m/s). Wilcoxon Signed Rank Test comparing before and after PWV values in the whole study group (N = 17) and split separately into IC (N= 8) & HV groups (N = 9)

**Multiple Regression Analysis PWV**

When included into a multiple regression model there were no statistically significant predictors of change in PWV values after RIPC (Table 15).

**Dependent Value: Post RIPC PWV**

| | Unstandardized Coefficients | | | | 95.0% Confidence Interval for B | |
|---|---|---|---|---|---|---|
| | Effect Estimate (B) | Std. Error | T Statistic | P Value | Lower Bound | Upper Bound |
| IC group | -1.516 | 2.316 | -0.655 | 0.529 | -6.755 | 3.723 |
| Pre RIPC PWV | 0.841 | 0.701 | 1.199 | 0.261 | -0.745 | 2.427 |
| Age (year) | 0.070 | 0.098 | 0.713 | 0.494 | -0.152 | 0.293 |
| Female Sex | -0.571 | 1.901 | -0.300 | 0.771 | -4.872 | 3.730 |
| diabetes | 0.785 | 2.890 | 0.272 | 0.792 | -5.753 | 7.323 |
| Systolic BP (mmHg) | -0.009 | 0.129 | -0.073 | 0.944 | -0.301 | 0.283 |
| Diastolic BP (mmHg) | 0.125 | 0.108 | 1.161 | 0.275 | -0.119 | 0.369 |
| (Constant) | -10.194 | 14.710 | -0.693 | 0.506 | -43.471 | 23.084 |

**Table 15** Multiple regression analysis to evaluate factors that impact arterial stiffness in response to RIPC, as measured by PWV(m/s). N= 17

### 11.3.4 Post Occlusive Reactive Hyperaemia

The peak perfusion units for PORH were calculated by subtracting the biological zero value from the peak post occlusive flow value.

**Spearman's Correlation**

There was a significant correlation between peak PU values before and after the RIPC protocol (Figure 19).

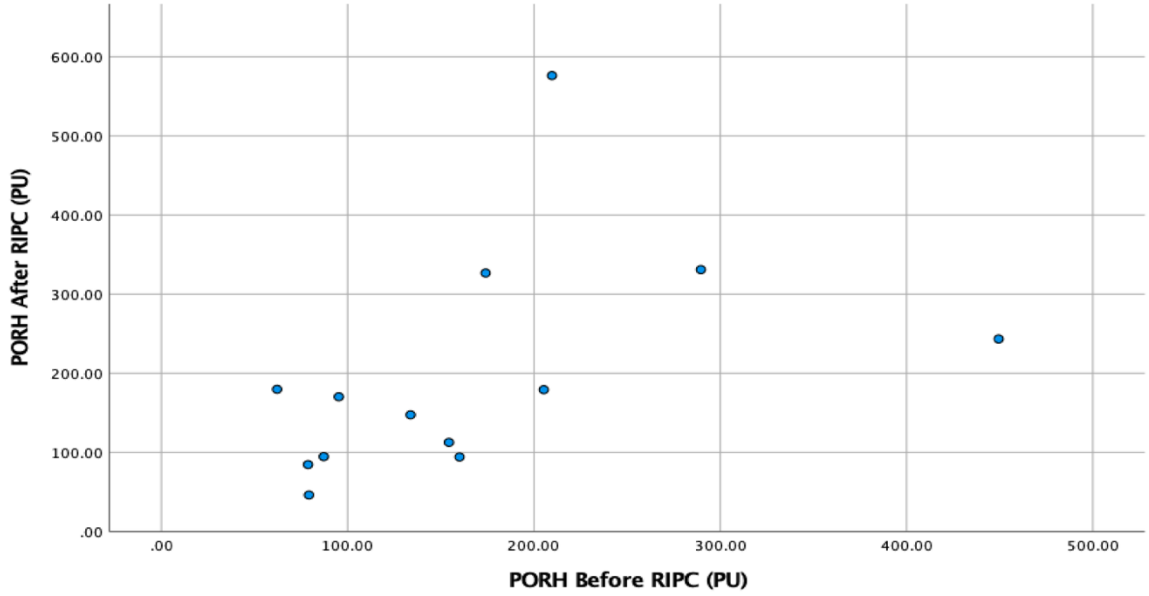



**Figure 19:** Scatter plot looking at correlation of PORH PU values before and after RIPC (Spearman's rho = 0.665, p = 0.013, *n*=13)

## Multiple Regression Analysis PORH

In order to assess whether being a healthy volunteer or participant with IC was predictive of post RIPC PORH scores we preformed regression analysis controlling for potential confounders (Table 16). None of the variables included in the model were significant.

| | Unstandardized Coefficients | | | | 95.0% Confidence Interval for B | |
|---|---|---|---|---|---|---|
| | Effect Estimate (B) | Std. Error | T statistic | P Value | Lower Bound | Upper Bound |
| PU Before RIPC | 0.724 | 0.775 | 0.934 | 0.393 | -1.268 | 2.716 |
| IC Group | 13.324 | 150.291 | 0.089 | 0.933 | -373.011 | 399.659 |
| age | 6.922 | 6.161 | 1.124 | 0.312 | -8.915 | 22.759 |
| Female Sex | 28.186 | 124.202 | 0.227 | 0.829 | -291.086 | 347.458 |
| Diabetes | -77.036 | 195.645 | -0.394 | 0.710 | -579.957 | 425.885 |
| Systolic BP | -7.130 | 7.393 | -0.964 | 0.379 | -26.135 | 11.875 |
| Diastolic BP | 0.555 | 8.347 | 0.067 | 0.950 | -20.902 | 22.012 |
| (Constant) | 588.393 | 1349.947 | 0.436 | 0.681 | -2881.757 | 4058.542 |

**Table 16** Multiple regression analysis to evaluate factors that impact endothelial function as determined by PORH in response to RIPC, as measured in PU. N= 13

# 12 DISCUSSION

The main aim of the study was to assess if repeated bouts of RIPC could improve endothelial function. Looking at the multiple regression analysis model for PWA it seems that HV have significantly lower arterial stiffness, as measured by AIx@75, compared to IC. This would suggest that the RIPC intervention may be having a greater effect on healthy volunteers. The most obvious differences between HV and IC participants will be level of endothelial dysfunction. Based purely on lack of clinical symptoms it could be assumed that the HV participants have superior endothelial function compared to IC. It may be that a healthy endothelium is more responsive to RIPC. Perhaps patients with IC, and by inference a degree of endothelial dysfunction, need more consecutive days of RIPC.

When devising the study protocol we used iontophoresis as a measure of endothelial function and tested only healthy volunteers. Despite seeing improvements in endothelial function with iontophoresis in HV perhaps we may not have seen such improvements if we had trialled the same protocol in IC participants. Our sample size was underpowered given the numbers that were recruited to the study. Based on previous studies in humans in RIPC(54) and from studies using iontophoresis and laser Doppler imaging (primary outcome measure)(71) future studies should aim to recruit 75 participants as this will give sufficient power at the 80% level with a probability of $P<0.05$ to detect significant before and after improvements in endothelial function.

Again, when devising the study protocol, we did see greater improvements in ACh mediated vasodilation in participants who completed seven days of RIPC compared to those who completed four days. It is possible that had IC participants, in the current study, completed a longer RIPC protocol of seven days we may have observed an improvement there also. The PWA results would seem to contradict other research looking at changes in arterial stiffness in healthy volunteers and those with CVD(54) where RIPC improved arterial stiffness in patients with CVD, who have a similar burden of disease to IC, and not in HV. Zagidullin et. al(54) also found with the improvement in arterial stiffness there was an improvement in systolic blood pressure suggesting an overall improvement in risk factors for CVD. In the current study arterial

stiffness was measured in a standardised and validated way which has been shown to be a surrogate measure of global endothelial function(64) however caution is required in presuming that our observed results are solely related to improvements in endothelial function. Arterial stiffness is also partly dependent on vasomotor tone which is mostly controlled by smooth muscle cells. This is independent of the endothelium but is related to NO(12) passing through the endothelial intima. As RIPC has been shown to increase bioavailability of NO this could partly explain the observed results. Although all of the other findings were not statistically significant there were trends in the data that warrant discussion.

**Iontophoresis**

Healthy volunteers again seem to have a greater improvement in endothelial-dependent ACh vasodilation compared to IC. This result did not reach statistical significance but looking at the data for response to endothelial-independent SNP mediated vasodilation there is much less of an improvement after the RIPC intervention. This would fit with previous studies that have found RIPC to have a targeted effect on the endothelium by increased production of NO(29). Regression analysis would seem to support this trend as well as it suggested, whilst controlling for confounding variables, that IC participants had reduced response to ACh mediated vasodilation compared to HV. Gender seems to be playing a role as being female resulted in greater improvements in endothelial function compared to males. A larger sample size is required to see if these trends would persist and if it would reach statistical significance.

Additionally, in future research, it would be of benefit to account for other variables that may be having an effect on results obtained. There is a strong association between diabetes and endothelial dysfunction and so measuring glucose levels could be of benefit. Studies have shown vasodilatory response to ACh iontophoresis is blunted in the presence of high glucose levels whether it be an acute rise or chronic disease(55)(72). In the current study we could have recorded patient BMI to examine if it had any effect on results. Obesity, as measured by very high BMI, is the excessive deposition of fat that can interfere with normal metabolic processes in the body. Increased accumulation of macronutrients on the adipose tissues can stimulate

production of proinflammatory mediators which can affect the healthy functioning of the endothelium(73)

**Post Occlusive Reactive Hyperaemia**

There were no significant changes conferred to before and after PORH values. It would seem that RIPC has no effect on PORH. RIPC is posited to effect the endothelium locally. The increased vasodilatory response when the occluded artery is released is primarily a mechanical stimulus (i.e occlusion of an artery with blood pressure cuff) and it is thought that this is partially dependent on myogenic-mediated vasodilation. It seems that RIPC has had no statistically significant effect on either. In a study looking at PORH in patients who have undergone seven days of RIPC there was an improvement in vascular reactivity as measured by PORH but this did not reach statistical significance(74). Perhaps as discussed participants should undergo seven days of RIPC although it does remain to be seen if this would produce a statistically significant change.

**Blood Markers**

Unfortunately, blood analyses were unable to be carried out due to the onset of the coronavirus pandemic. It would have been useful to have these results as it would have allowed for analysis of correlations between plasma levels of markers of vascular function and changes seen in microvascular and macrovascular assessments. TNF-α was one of the main cytokines we intended to study. As discussed in the introduction TNF-α can be proinflammatory but may also play a role in the SAFE pathway when initiated by RIPC, dependent on its binding to the TNF receptor 2. Increased levels of IL-1β & IL-6 have been associated with impaired endothelial function and increased risk of cardiovascular events(75) IL-10 has a protective effect on endothelial function however both IL-6 and IL-10 have a role in some of the protective effects seen in RIPC. It is thought that both IL-6 and IL-10 are both activated via STAT3 and have been shown to be associated with reduced infarct size in RIPC participants(76)

## 12.1 LIMITATIONS OF STUDY

The main limitation of the study is the sample size. With 17 participants any data gathered would be unlikely to reach statistical significance. In general when splitting the sample group into HV and IC the two groups were not well matched in terms of age or other risk factors such as smoking. None of the HV were smokers or diabetic. For the purposes of the study it was felt that this was an appropriate mismatch. Participants with no clinical symptoms of atherosclerotic disease can be assumed to have a healthier endothelium than the IC participants. HV participants were younger which again suggests healthier endothelium. One of the main aims of the study was to assess the effects of RIPC endothelial function and so there is benefit in examining its effect on healthy endothelium and diseased endothelium.

The study did not employ a placebo group. Having a placebo group would allow better analysis of the RIPC protocol and allow to control for confounding factors that may affect study results such as smoking, exercising or taking on food or drink (especially caffeine) prior to assessment.

Compliance with the RIPC protocol may have been poor. Carrying out the protocol required using a blood pressure cuff at home for four consecutive days. There is a possibility that less motivated participants may not have completed the protocol in its entirety or may have partially completed it. It was impossible to control for this as the resources were not available to have patients attend the lab each day to be observed completing the RIPC protocol.

When performing iontophoresis, the skin over the anterior compartment of the lower leg was used for analysis. The rationale for this was that as patients with claudication are generally affected by atherosclerotic disease in the arteries supplying the lower limbs it would be of benefit to measure microvascular response over this area. However, the maximal penetration of iontophoresis is about 1-1.5mm(56). The level of drug penetration can be affected by differences in skin composition between participants. The dermal layer in the leg tends to be deeper than in the arm. With RIPC the potential improvements should be conferred to remote sites. If these

improvements are observed in the microvascular bed of the arm then it could be inferred that there is a similar improvement in the lower leg arteries.

When analysing PORH there were data for 13 participants. In two cases this was due to machine software failure and in the other two the participants were unable to tolerate 5 minutes of cuff occlusion. A complete case analysis was performed, and as a result, patients with any missing data were excluded. This is an acceptable approach when there are no systematic differences between patients with complete and incomplete data.

## 12.2 SUGGESTIONS FOR FUTURE RESEARCH

Future research should focus on ensuring adequate sample size so that the study is powered appropriately. In terms of recruiting participants and compliance with the protocol perhaps with the current increased use of Microsoft Teams and Zoom meetings it may be of benefit for participants to have an online conference appointment with researchers to ensure that they are managing with the RIPC protocol. This would certainly be convenient for the participants but obviously would be more time consuming for researchers. It would, however, ensure that the protocol is being followed as intended.

As there is still uncertainty as to what length of RIPC protocol is of greatest benefit when examining differences in endothelial function in IC and HV. Will better results be obtained in those who undergo seven days of RIPC as opposed to four days. A paper published after the current study concluded has shown that seven consecutive days of RIPC does produce a statistically significant difference in SNP mediated vasodilation, via iontophoresis, compared to baseline(77). The same study however did not find any difference between a seven-day RIPC protocol and a fourteen-day protocol suggesting there may be a saturation point to repeated bouts of RIPC.

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# 14 APPENDIX 1

## 14.1 RAW DATA FROM STUDY

### Baseline Characteristics

| Pt ID | Group | sex | age | smoking | diabetes | Systolic BP | Diastolic BP |
|---|---|---|---|---|---|---|---|
| 1 | HV | Female | 57.00 | Non | Non | 130.00 | 80.00 |
| 2 | IC | Male | 57.00 | Smoker | Non | 135.00 | 88.00 |
| 3 | HV | Male | 40.00 | Non | Non | 124.00 | 88.00 |
| 4 | IC | Female | 56.00 | Smoker | Non | 135.00 | 78.00 |
| 5 | IC | Male | 81.00 | Smoker | Non | 124.00 | 64.00 |
| 6 | HV | Male | 64.00 | Non | Non | 140.00 | 66.00 |
| 7 | HV | Male | 40.00 | Non | Non | 112.00 | 82.00 |
| 8 | IC | Male | 60.00 | Smoker | Diabetic | 117.00 | 64.00 |
| 9 | IC | Male | 62.00 | Smoker | Diabetic | 124.00 | 81.00 |
| 10 | HV | Female | 64.00 | Non | Non | 150.00 | 74.00 |
| 11 | IC | Male | 60.00 | Smoker | Non | 155.00 | 95.00 |
| 12 | IC | Male | 75.00 | Smoker | Non | 132.00 | 85.00 |
| 13 | IC | Male | 58.00 | Smoker | Non | 126.00 | 82.00 |
| 14 | HV | Female | 30.00 | Non | Non | 117.00 | 82.00 |
| 15 | HV | Female | 30.00 | Non | Non | 125.00 | 82.00 |
| 16 | HV | Male | 30.00 | Non | Non | 119.00 | 71.00 |
| 17 | HV | Male | 30.00 | Non | Non | 108.00 | 61.00 |

**Pulse Wave Velocity and Pulse Wave Analysis**

| Pt ID | Group | PWV.pre | PWV.post | PWV.diff | PWA.pre | PWA.post | PWA.diff |
|---|---|---|---|---|---|---|---|
| 1 | HV | 6.40 | 5.90 | -0.50 | 38.00 | 28.00 | -10.00 |
| 2 | IC | 9.70 | 8.70 | -1.00 | 34.00 | 49.00 | 15.00 |
| 3 | HV | 8.90 | 9.40 | 0.50 | 38.00 | 32.00 | -6.00 |
| 4 | IC | 7.10 | 5.80 | -1.30 | 37.00 | 41.00 | 4.00 |
| 5 | IC | 13.30 | 9.20 | -4.10 | 43.00 | 38.00 | -5.00 |
| 6 | HV | 7.80 | 8.10 | 0.30 | 30.00 | 12.00 | -18.00 |
| 7 | HV | 10.90 | 8.80 | -2.10 | 11.00 | 13.00 | 2.00 |
| 8 | IC | 8.20 | 8.00 | -0.20 | 24.00 | 20.00 | -4.00 |
| 9 | IC | 10.10 | 10.00 | -0.10 | 35.00 | 35.00 | 0.00 |
| 10 | HV | 6.10 | 6.90 | 0.80 | 35.00 | 21.00 | -14.00 |
| 11 | IC | 8.80 | 9.60 | 0.80 | 45.00 | 35.00 | -10.00 |
| 12 | IC | 11.40 | 19.50 | 8.10 | 79.00 | 50.00 | -29.00 |
| 13 | IC | 9.30 | 7.70 | -1.60 | 45.00 | 44.00 | -1.00 |
| 14 | HV | 10.10 | 10.40 | 0.30 | -7.00 | -10.00 | -3.00 |
| 15 | HV | 7.60 | 7.10 | -0.50 | 10.00 | 13.00 | 3.00 |
| 16 | HV | 7.40 | 7.20 | -0.20 | 2.00 | -1.00 | -3.00 |
| 17 | HV | 8.80 | 6.80 | -2.00 | 1.00 | 1.00 | 0.00 |

**Iontophoresis – Ach**

| Pt ID | Group | ach.b.base | ach.b.peak | ach.diff.b | ach.a.base | ach.a.peak | ach.diff.a | Difference PU ACH |
|---|---|---|---|---|---|---|---|---|
| 1 | HV | 32.00 | 278.00 | 246.00 | 59.00 | 420.00 | 361.00 | 115.00 |
| 2 | IC | 131.00 | 423.00 | 292.00 | 69.00 | 138.00 | 69.00 | -223.00 |
| 3 | HV | 26.00 | 63.00 | 37.00 | 91.00 | 451.00 | 360.00 | 323.00 |
| 4 | IC | 52.00 | 337.00 | 285.00 | 21.00 | 594.00 | 573.00 | 288.00 |
| 5 | IC | 16.00 | 257.00 | 241.00 | 22.00 | 55.00 | 33.00 | -208.00 |
| 6 | HV | 39.00 | 289.00 | 250.00 | 44.00 | 264.00 | 220.00 | -30.00 |
| 7 | HV | 25.00 | 157.00 | 132.00 | 6.00 | 237.00 | 231.00 | 99.00 |
| 8 | IC | 26.00 | 216.00 | 190.00 | 50.00 | 82.00 | 32.00 | -158.00 |
| 9 | IC | 35.00 | 80.00 | 45.00 | 9.00 | 46.00 | 37.00 | -8.00 |
| 10 | HV | 24.00 | 398.00 | 374.00 | 95.00 | 183.00 | 88.00 | -286.00 |
| 11 | IC | 23.00 | 34.00 | 11.00 | 33.00 | 53.00 | 20.00 | 9.00 |
| 12 | IC | 78.00 | 127.00 | 49.00 | 32.00 | 403.00 | 371.00 | 322.00 |
| 13 | IC | 13.00 | 306.00 | 293.00 | 28.00 | 437.00 | 409.00 | 116.00 |
| 14 | HV | 26.00 | 299.00 | 273.00 | 39.00 | 501.00 | 462.00 | 189.00 |
| 15 | HV | 0.00 | 215.00 | 215.00 | 9.00 | 244.00 | 235.00 | 20.00 |
| 16 | HV | 33.00 | 193.00 | 160.00 | 21.00 | 188.00 | 167.00 | 7.00 |
| 17 | HV | 27.00 | 164.00 | 137.00 | 11.00 | 113.00 | 102.00 | -35.00 |

**Iontophoresis – SNP**

| Pt ID | Group | snp.b.base | snp.b.peak | snp.diff.b | snp.a.base | snp.a.peak | snp.diff.a | Difference Flux SNP |
|---|---|---|---|---|---|---|---|---|
| 1 | HV | 29.00 | 423.00 | 394.00 | 55.00 | 413.00 | 358.00 | -36.00 |
| 2 | IC | 248.00 | 256.00 | 8.00 | 75.00 | 219.00 | 144.00 | 136.00 |
| 3 | HV | 32.00 | 63.00 | 31.00 | 38.00 | 188.00 | 150.00 | 119.00 |
| 4 | IC | 49.00 | 386.00 | 337.00 | 44.00 | 192.00 | 148.00 | -189.00 |
| 5 | IC | 23.00 | 211.00 | 188.00 | 17.00 | 110.00 | 93.00 | -95.00 |
| 6 | HV | 28.00 | 168.00 | 140.00 | 49.00 | 253.00 | 204.00 | 64.00 |
| 7 | HV | 34.00 | 223.00 | 189.00 | 3.00 | 196.00 | 193.00 | 4.00 |
| 8 | IC | 24.00 | 154.00 | 130.00 | 51.00 | 73.00 | 22.00 | -108.00 |
| 9 | IC | 66.00 | 89.00 | 23.00 | 40.00 | 64.00 | 24.00 | 1.00 |
| 10 | HV | 58.00 | 258.00 | 200.00 | 54.00 | 99.00 | 45.00 | -155.00 |
| 11 | IC | 32.00 | 76.00 | 44.00 | 20.00 | 87.00 | 67.00 | 23.00 |
| 12 | IC | 60.00 | 314.00 | 254.00 | 165.00 | 438.00 | 273.00 | 19.00 |
| 13 | IC | 11.00 | 34.00 | 23.00 | 1.00 | 151.00 | 150.00 | 127.00 |
| 14 | HV | 4.00 | 53.00 | 49.00 | 33.00 | 395.00 | 362.00 | 313.00 |
| 15 | HV | 0.00 | 78.00 | 78.00 | 6.00 | 121.00 | 115.00 | 37.00 |
| 16 | HV | 0.00 | 151.00 | 151.00 | 0.00 | 80.00 | 80.00 | -71.00 |
| 17 | HV | 15.00 | 132.00 | 117.00 | 7.00 | 7.00 | 0.00 | -117.00 |

**Reactive Hyperaemia**

| Pt ID | Group | BioZeroBefore | PeakPUBefore | True PU Before RIPC | BioZeroAfter | PeakPUAfter | True PU After RIPC |
|---|---|---|---|---|---|---|---|
| 1 | HV | 30.00 | 204.10 | 174.10 | 47.50 | 374.20 | 326.70 |
| 2 | IC | 24.80 | 103.60 | 78.80 | 25.60 | 110.20 | 84.60 |
| 3 | HV | 30.00 | 184.40 | 154.40 | 35.90 | 148.60 | 112.70 |
| 4 | IC | 32.80 | 120.00 | 87.20 | 29.60 | 124.30 | 94.70 |
| 5 | IC | | | | | | |
| 6 | HV | 34.70 | 130.00 | 95.30 | 19.70 | 190.00 | 170.30 |
| 7 | HV | | | | | | |
| 8 | IC | 20.00 | 309.60 | 289.60 | 30.00 | 361.00 | 331.00 |
| 9 | IC | 42.80 | 105.00 | 62.20 | 30.00 | 209.80 | 179.80 |
| 10 | HV | | | | | | |
| 11 | IC | 30.10 | 109.40 | 79.30 | 40.00 | 86.20 | 46.20 |
| 12 | IC | 51.20 | 185.00 | 133.80 | 48.50 | 196.00 | 147.50 |
| 13 | IC | 29.00 | 238.70 | 209.70 | 23.80 | 600.00 | 576.20 |
| 14 | HV | 54.70 | 260.00 | 205.30 | 46.10 | 225.40 | 179.30 |
| 15 | HV | 29.90 | 190.00 | 160.10 | 25.80 | 120.00 | 94.20 |
| 16 | HV | 99.00 | 548.40 | 449.40 | 102.20 | 345.60 | 243.40 |
| 17 | HV | | | | | | |